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THIS IS TO CERTIFY THAT THE THESIS PREPARED UNDER MY SUPERVISION BY

Mark Eugene Duban

ENTITLED Methods to Produce and Physically Characterize Compositionally

Asymmetric Phospholipid Vesicles Unbiased by Small Radius of Curvature

IS APPROVED BY ME AS FULFILLING THIS PART OF THE REQUIREMENTS FOR THE

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METHODS TO PRODUCE AND PHYSICALLY CHARACTERIZE COMPOSITIONALLY ASYMMETRIC PHOSPHOLIPID VESICLES UNBIASED BY SMALL RADIUS OF CURVATURE

BY

MARK EUGENE DUBAN

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Abbreviations

A	absorbance, wavelength indicated in subscript
DISP	vesicles by dispersion
DMPC	O-(1,2-dimyristoyl-*sn* -glycero-3-phosphoryl)choline
DPH	1,6-diphenyl-1,3,5-hexatriene
EDTA	(ethylenedinitrilo)tetraacetic acid
EG	ethylene glycol
EGTA	ethylenebis(oxyethylenenitrilo)tetraacetic acid
FID	in NMR, the free induction decay
FT	Fourier transform
GFC	gel filtration chromatograohy
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	high performance (pressure) liquid chromatography
ILR	inner leaflet resonance
laurodan	6-lauroyl-2-dimethylaminonaphthalene
MES	2-(N-morpholino)ethanesulfonic acid
N-NBD-	N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-
NMR	high resolution Fourier transform nuclear magnetic resonance
NOE	in NMR, the nuclear Overhauser effect
NOEE	nuclear Overhauser effect enhancement
OLR	outer leaflet resonance
PA	O-1,2-diacyl-*sn* -glycero-3-phosphatidic acid
PAGE	polyacrylamide gel electrophoresis
PC	O-(1,2-diacyl-*sn* -glycero-3-phosphoryl) choline; phosphatidylcholine
PE	O-(1,2-diacyl-*sn*-glycero-3-phosphoryl) ethanolamine; phosphatidylethanol-amine
PHLD	phosphatidylcholine phosphohydrolase, EC 2.1.8.8; phospholipase D
PPR	in NMR, praesodymium-to-phospholipid ratio
PIPES	piperazine-N, N'bis(2-ethanesulfonic acid)
SDS	dodecyl sulfate, sodium salt
TES	2-[tris(hydroxymethyl)methylamino]-1-ethanesulfonic acid
TLC	thin layer chromatography
TMA-DPH	1-[4-(trimethylammonio)phenyl]-6-phenyl]-1,3,5-hexatriene
Tricine	N-[tris(hydroxymethyl)methyl] glycine
Tris	tris(hydroxymethyl)aminomethane
VBI	vesicles by injection
VBS	vesicles by sonication

The following parameter abbreviations appear in the figure legends of NMR spectra in Results and Discussion; all are defined at greater length in the text of the Methods section:

AK	scaling factor
AT	acquisition time
DE	pre-acquisition delay
D5	post-acquisition
DP	data points, number real/imaginary in frequency domain
NA	number of acquisitions
PW	pulse width
SW	sweep width

Symbols

ϵ	$1 - K_{ave}$
ϵ	extinction coefficient
erf-1	inverse error function
I_{par}, I_{perp}	fluorsence intensity parallel and perpendicular to the incident plane
K_{ave}	equilibrium constant for particle distribution between gel pore and fluid phase
P	fluorescence polarization
r	fluorescence anisotropy
T_c	temperature of phospholipid gel to liquid crystalline phase transition
T_c+10°C	temperaure ten degrees greater than the highest phospholipid T_c (of phospholipids in a mixture)
$V_{1/2}$	peak width at half height, in units of volume
V_e, V_o, V_t	in GFC: elution, total and void volumes, respectively

Abstract

A program of experiments was designed to answer several fundamental questions regarding the nature of the observed asymmetry in the phospholipid component of biological membranes. Vesicles of phosphatidylcholine were routinely produced by slow injection of ethanolic phosphatidylcholine into an aqueous buffer, followed by removal of ethanol and clarification by centrifugation. The vesicles by injection were shown to be (i) free of preparative impurities by gel filtration and standard analytical methods, (ii) a population with fairly narrow distribution about a mean diameter of ≈90 nm by gel filtration on Sephacryl S1000, and (iii) unilamellar and sealed by phosphorus-31 nuclear magnetic resonance spectroscopy (^{31}P NMR) in the presence of the line-shift reagent praesodymium (III) chloride.

Hydrolytic cleavage of phosphatidylcholine in the outer leaflet of vesicles to phosphatidic acid and free choline was catalyzed by the phosphatidylcholine phosphohydrolase (phospholipase D) from Savoy cabbage. Optimum conditions for hydrolysis were determined by series of experiments varying the substrate form, purity of the enzyme preparation, pH and calcium concentration. The hydrolysis of vesicles catalyzed by partially-purified enzyme at pH 6.0 in the absence of exogenously added calcium resulted in a stable population of vesicles composed of ≈30 mol% phosphatidic acid. The product vesicles were separated from free choline and from enzyme by gel filtration on Sephadex G200 and were shown to be free of any lipid-soluble byproduct or oxidative impurity by standard analytical methods. Under appropriate reaction conditions these vesicles appeared to be of comparable integrity to the original substrate population.

Control ^{31}P NMR experiments performed on model compounds and on vesicles by sonication and injection suggested that ^{31}P{^{1}H} nuclear Overhauser effects normally observed in phospholipid bilayers were eliminated by the application of a standard gated proton decoupling sequence. ^{31}P NMR experiments involving titration with the shift reagent praesodymium (III) chloride (or exchange with reagent-containing buffers) were successfully used to determine conditions for resolving the phosphorus resonances attributable to phospholipids in the inner and outer leaflets of vesicles prepared by injection (≈90 nm) and of vesicles prepared by sonication (≈20 nm). Preliminary NMR experiments on sonicated phosphatidylcholine/phosphatidic acid vesicles indicated that future experiments aimed at measuring the amount of outer leaflet phosphatidic acid should be performed with encapsulated rather than exogenously-added shift reagent.

Fluorescence polarization experiments were performed using the hydrophobic probe 1,6-diphenyl-1,3,5-hexatriene (DPH) and the amphiphilic ("anchored") probe 1-[4-(trimethylammonio)-phenyl]-6-phenyl]-1,3,5-hexatriene (TMA-DPH). The standard procedures to produce vesicles by injection and by sonication were used, with phospholipid concentrations chosen to produce vesicles with defined phospholipid symmetry or asymmetry. The results indicate that while DPH is generally more sensitive than TMA-DPH to the temperature dependence of changes in probe environment, it is much less sensitive to the structural and dynamic differences reported by TMA-DPH in phosphatidic acid-containing vesicles. The effect of the presence of phosphatidic acid was more pronounced in large diameter vesicles than in small. Limited data suggest that symmetric and asymmetric distributions of the same concentration of phosphatidic acid result in different membrane properties.

The synthesis of 2,6-disubstituted naphthalenes was considered as a source for neutral amphipathic membrane probes. The fluorescence emission spectra of laurodan, the 6-lauroyl analog of the dipole moment-sensitive fluorophore 6-propionyl-2-dimethylaminonaphthalene (prodan), were measured in a variety of solvents. Emission maxima were found to be equally sensitive to solvent polarity, and slightly blue-shifted relative to prodan. Synthetic schemes were outlined to produce amphiphilic laurodan analogs using hexoses as non-charged polar "anchors." A scheme was devised using diaminoalkanes as hydrophobic "spacers" so that the dipole moment-sensitive fluorescent acylnaphthyl moiety could be immersed to varying degrees in the hydrophobic core of the membrane. The use of such probes in characterizing bilayers containing asymmetric distributions of anionic phospholipids is discussed.

I. Introduction

A. Biological Membranes as a Fluid Mosaic

The membranes which encompass cells of living organisms and which form their interior compartments are generally understood to be mosaics of protein immersed in or associated with fluid bilayers of lipid. This paradigm was proposed by S.J. Singer and G.L. Nicholson in 1972 based on observations in their laboratories and in laboratories of their contemporaries (172, 173).

Much of the work establishing the fluid-mosaic nature of biological membranes was aimed at discerning the relationship between the phospholipid and protein components of the erythrocyte plasma membrane. Studies in preceding decades had firmly established that a bilayer of lipid was responsible for the barrier properties of the membrane. Contemporary work included (i) circular dichroism experiments, revealing a degree of protein alpha-helical content consistent with a folded, globular form for membrane proteins, and (ii) freeze-fracture electron microscopic studies, presenting particle-studded images consistent with the proposition that the globular proteins were embedded in the lipid bilayer matrix (75, 172). Whole cell studies of the mixing of distinct antigens on the surface of a fusion heterocaryon and of the distribution of erythrocyte membrane proteins provided evidence that the bilayer matrix was fluid in nature and without long-range order of the protein component (173, 59).

The structural features presented in this early work continue to be the object of basic research. Much of the research seeks to extend the paradigm by increasing our understanding of the relationship between structure and the physical and dynamic properties of biological membranes (cf. 178, here and following). Included are studies of whole cells, isolated membrane preparations and of model membrane systems composed of natural or synthetic lipid. Often the focus is on the interaction of an individual molecular species with like or unlike species, e.g., in studies of lipid cooperativity and of lipid-protein interactions. Other studies focus on microscopic phenomena such as cellular aggregation and membrane fusion, where the aim is to discern detailed molecular mechanisms. A recent monograph by R.B. Gennis (62) presents a thorough account of the progress that has been made in our understanding of the structure and function of biological membranes. A recent shorter work by P. Yeagle (210) describes in particular the progress in our understanding of the structure and function of the phospholipid component of membranes.

B. A Fluid Mosaic of Symmetric and Asymmetric Domains

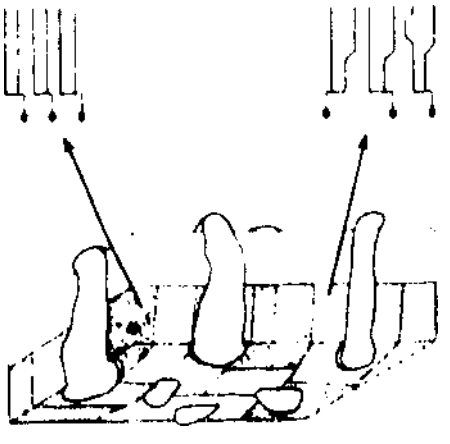
In more recent studies, two interesting molecular features have been elaborated. First, it has become clear that almost all components of biological membranes are *vectorally* or *asymmetrically* distributed (62, 210). This understanding is consistent with the observation that such membrane-associated functions as transport and phospholipid biosynthesis are typically vectoral in nature (172). Studies in this laboratory are among those which indicate that such an asymmetric distribution might have physiologic consequence: Clancy *et al.* note that the apparent K_m of an anionic substrate of the integral membrane protein D-β-hydroxybutyrate dehydrogenase increases four to ten fold when assayed in membranes with elevated levels of asymmetrically distributed anionic phospholipid (refs. 37, 204 and Results and Discussion).

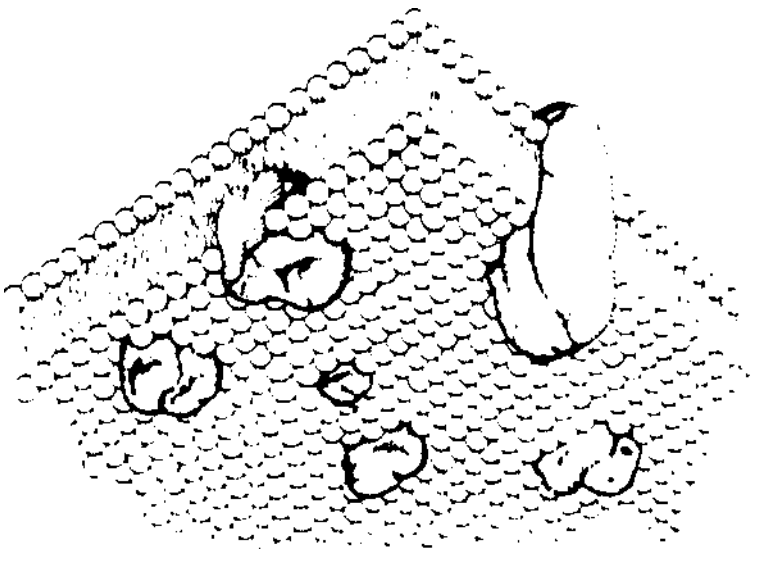
The second molecular feature that has been elaborated is referred to as *boundary lipid,* i.e., the notion that some proteins within membranes sequester particular phospholipids through hydrophobic and other interactions (62, 210). This understanding is consistent with such observations as the specific and absolute requirement of certain membrane-bound enzymes for a noncovalently-associated phospholipid species (155, 36 and references therein). Studies from this laboratory on this molecular feature include kinetic measurements on such a phospholipid-requiring enzyme, fluorescence measurements on reconstituted lipid-protein systems and whole cell studies involving the budding of membrane-coated viruses (28, 36, 37, 73).

Taken synoptically, these and other studies of membrane symmetry and protein-lipid interaction support and clarify the presentation of biological membranes as a fluid mosaic structure. Moreover, this more recent work suggests that the mosaic of lipid and protein domains of the outer leaflet might differ from the mosaic of the domains of the inner leaflet. Consider as an analogy the ornate pattern of cloth shapes in a patchwork quilt. Each side of the quilt represents the domain structure of one leaflet of the plasma membrane at an instant in time (see Figure 1 and refs. 62, 112). Just as the design of the patchwork quilt might include a lack of correspondence between patterns at any point on the two sides of the quilt, so the design of the plasma membrane might include functionally significant points of protein and phospholipid asymmetry in a membrane where phospholipid is apparently symmetrically distributed.

The complexity of this asymmetric patchwork is only infrequently addressed in studies of the physical properties of membranes, e.g. the rotational and translational mobilities of membrane components, the characteristics of their contributions to the phase behavior of the membrane, etc. It is known that each membrane component contributes to the measurable physical properties of the membrane (62, 36), and it is reasonable to assume that asymmetries of individual components

Figure 1 Representations of the fluid mosaic model of membrane structure. Representations of the fluid mosaic model are from the text of Gennis (A, ref. 62) and a report from the laboratories of Kleinfeld and Karnovsky (B, ref. 98). The Gennis graphic originates in the laboratory of S.J. Singer, and emphasizes the varying degree to which membrane proteins are immersed in the phospholipid bilayer. The Kleinfeld and Karnovsky graphic emphasizes a non-uniform, domain-like structure for one leaflet of the bilayer. This graphic notes the potential for segregation of phospholipids with unsaturated (left arrow) and saturated (right arrow) acyl chains into domains with consequent differences in fluidity (98). The possible presence of a different "patchwork" of domains on the opposing leaflet is inferred.





would likewise affect these properties. Reports of experiments aimed at determining the contribution of the asymmetries of individual components to the bulk physical properties of membranes are unfortunately scarce (148, 210).

In the process of this laboratory's study of the nature of the interactions between components of biological membranes, a means of performing such a series of experiments has presented itself (36, 37). The contribution to be examined is that of a low level of acidic phospholipid (phosphatidic acid, PA) in a model phosphatidylcholine (PC) membrane system. The rest of the introduction, then, is a review of (i) available data on phospholipid asymmetry in biological and model membranes, noting especially the studies involving PC/PA systems, and (ii) experimental considerations in the choices of a model membrane system, a method for inducing the asymmetric distribution of phospholipid and a means of assessing the structural and dynamic effects of such an induced asymmetry.

C. Asymmetry of the Phospholipid Component of Biological Membranes

The distribution of phospholipid across the bilayer plane has been studied in a wide variety of normal and pathological mammalian plasma membranes, in all isolatable eukaryotic subcellular organelles and in many prokaryotic and archaebacterial membranes. In addition to cataloging the growing number of studies of lipid distributions, reviewers in this area (cf. Bergleson and Barsukov, ref. 17, Op den Kamp, ref. 135 and Thompson, ref. 185) address questions of the origin, maintainance and functional significance of, and to a limited extent the altered physical properties imparted by an asymmetric distribution of the phospholipids. Effort has been directed toward understanding the role played by cytoskeletal proteins, especially those of the erythrocyte, in the maintainance of an asymmetric distribution of phospholipid. Work also focuses on the involvement of asymmetry in such relevant phenomena as pathologies of the erythrocyte and other

erythrocyte and other cells (cf. 154, 164), the modulation of the activity of integral membrane proteins (13, 139), the recognition of idiotypes by the immune system (108), the production of such messengers as platelet activating factor (150) and development of erythropoid cells (149). Recently, the oxidative collapse of phospholipid asymmetry in the nerve cell was linked to the deterioration of celluiar processes with aging, a proposal which stimulated a lengthy literature discussion (see Schroeder, ref. 162, and other reports in the same journal issue).

An abbreviated summary of the reported asymmetries of phospholipids in the biological membranes of eukaryotes is presented in Table 1. The nature of the distribution of complex lipids (such as glycolipids) and of many phospholipids across the bilayer of the erythrocyte plasma membrane is well established. The distributions of cholesterol and other neutral lipids are not so well established, though model membrane and erythrocyte work has been performed (83). In the erythrocyte and perhaps most mammalian plasma membranes the concentration of the choline phospholipids PC and sphingomyelin are higher in the outer leaflet, where all complex lipid (e.g., glycolipid) is located (135, 185). In fact, sphingomyelin is found almost exclusively in the outer leaflet of erythrocytes (135). Phosphatidylethanolamine (PE) and phosphatidylserine are localized preferentially in the inner leaflet of most mammalian cells that have been studied (135).

The symmetry characteristics of phospholipids in subcellular membranes are less well established; the asymmetries noted are yet subject to debate. It is known that the side of the inner mitochondrial membrane facing the cytoplasm is enriched in PC, while the matrix side of that membrane is enriched with PE and cardiolipin (130). Studies of the symmetry of the endoplasmic reticulum, the Golgi apparatus and isolated microsomal membranes are the matter of some controversy, with discussion focusing on suggested artifacts of the methods used for determining the transbilayer phospholipid distribution (79, 80, 190, 191).

Table 1 Phospholipid Asymmetries in the Biological Membranes of Eukaryotic Cells.[a]

System[b]	Leaflet enrichment[b]		Relatively symmetric phospholipid(s)	Ref.
	Inner	Outer		
erythrocytes				
normal	PE, PI, PS	PC, SPH	--	11, 154
sickle, oxy	PE, PI, PS	PC, SPH	--	154
sickle, hyp	PI	PS, SPH	PE, PC	154
other plasma membranes				
LM cell	PE, PI, PS	SPH	--	135, 17
LM cell	unsat PL	sat PL	--	135
Friend cell	PS	SPH	PC, PE, PI	150
Krebs ascites PL	1-alkyl,2-acyl	1,2-diacyl	--	150
Krebs ascites	PS, CL	--	PC, PE, PI, SPH	11
platelet	PE, PS, PI	SPH	--	135, 150
brush border	PC, PE	--[c]	--	135
outer rod	PC	PS, PE	--	11
subcellular membranes				
inner mitochondrial	PE, CL	PC	--	135
endoplasmic reticulum	PC	PE	--	17
sarcoplasmic reticulum	PS	PE	PC	135

[a]Reported symmetries and asymmetries are either the consensus of reports, or are based on a single report that is uncontested and apparently reliable. If a report of the asymmetry of a particular species in a membrane remains controversial, it was not included in the listing for that membrane. The list of phospholipids reported within each entry is therefore not necessarily a complete description of the phospholipid composition of the membrane.

[b]Non-standard abbreviations: oxy - under conditions of adequate oxygen tension, hyp - under conditions of hypoxia, PL - phospholipid, sat - PL containing saturated fatty acids, unsat - PL containing unsaturated fatty acids.

[c]Unusual in that its outer leaflet is composed of an extremely high percent (w/w) of protein.

E. Phospholipid Asymmetry in Model Membranes

In order to simplify the study of membrane asymmetry, and of other important membrane-associated phenomena, researchers have turned to the study of vesicle membrane systems (cf. refs. 42 and 181 for reviews). Of the possible systems for study, multilamellar vesicles of the sort introduced by Bangham *et al.* in 1966 (9) and the sonicated, minimal diameter unilamellar vesicle introduced by Huang in 1969 (82) have been the primary contemporary models, though large diameter unilamellar vesicles are growing in popularity.[1] Unfortunately, almost all research done on phospholipid asymmetry in model systems has been performed on the Huang-type vesicles by sonication (VBS). Such vesicles have been termed "compositionally asymmetric" as the phospholipids in two component systems distribute asymmetrically during the process of sonication (185, 186).

In these structures, where the ratio of phospholipid in the outer leaflet is roughly twice that of the inner leaflet, the orientations of different phospholipid species across the membrane are determined almost exclusively by headgroup packing constraints (62, 82, 116, 210). For example, VBS of equimolar amounts of PC and PE, PA or phosphatidylinositol are found to have the non-PC phospholipid preferentially localized in the inner leaflet of the vesicle (185, 186). When extracted phospholipid from erythrocytes are used to form the small diameter vesicles, the same localizations

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[1] Vesicles with a wide variety of physical characteristics can be formed as combinations of protein and lipid and conditions of hydration are varied. After composition, the structures produced can be classified most simply according to apparent diameter and to the number of concentric bilayers (lamellae) that are formed during hydration. Generally, three classes of vesicles are recognized and variously defined, in the literature: large multilamellar (LMV or, more commonly, MLV), small unilamellar (SUV) and large unilamellar (LUV). Physical properties of a particular preparation of vesicles are often reported with assignment of one of these labels, but without actual characterization of the vesicle according to diameter or number of lamellae. Most often, assignment is actually based on a prior published characterization of a more-or-less comparable preparation. Here vesicle populations are described and abbreviated in a manner which communicates the methods used in their preparation; hence, DISP are vesicles by dispersion, and VBS and VBI are vesicles by sonication and injection, respectively. Any further characterization with respect to apparent diameter and number of lamellae that is performed is stated explicitly.

are reported (104). The case for phosphatidylserine is somewhat more complex in that its location varies with pH of the buffering media (14). As a general rule, however, the size of the phospholipid headgroup has been shown to be the primary determinant of the location of a lipid species across the membrane of sonicated preparations (Table 2 and references cited therein).

There has been a single study comparing VBS with larger diameter unilamellar vesicles: in preparations of VBS and of vesicles prepared by ethanol injection (VBI), Nordlund *et al.* (131) demonstrated that the 10 mol% PE included was distributed symmetrically in the larger diameter PC vesicle. In the small vesicle it was again found almost exclusively on the inner leaflet. Based on these and other results, the authors of this study state that small vesicles are poor models for the study of the equilibrium processes giving rise to membrane asymmetry. Unfortunately, this type of careful comparative work has not been performed for any other lipid species.

E. Experimental Considerations

1. The Choice of a Model Membrane System

The report of Nordlund *et al.* describes populations of small, asymmetric and large symmetric two component phospholipid vesicles. The question of the independent variation of the physical properties of the vesicles with vesicle size and symmetry could not therefore be addressed. In studies focusing on the variation of physical properties with vesicle diameter, differences between VBS and larger diameter vesicles have been noted. Such biologically relevant bilayer properties as osmotic activity, protein association and susceptibility to aggregation and fusion have been noted to differ (116, 151). Moreover, differences in spectroscopic (fluorescence, magnetic resonance, infrared and Raman) and calorimetric parameters indicative of the packing and motion of bilayer phospholipids have been shown to vary with the radius of the vesicle studied (21, 44, 83, 150,

Table 2 Consensus Phospholipid Asymmetries in Vesicles by Sonication (VBS) and by Other Methods.[a]

Method of Preparation[b] Composition	Leaflet enrichment[c] Inner	Leaflet enrichment[c] Outer	Analytical Method and conditions[d]	Ref.
Vesicles by sonication (VBS)				
PC/PE	PE	PC	by TNBS, NMR	117,131
PC/PS	PS	PC	by NMR, pH < 8	14
	--	--	by NMR, pH 8-9	14
	PC	PS	by NMR, pH > 9	14
PC/PI	PI	PC	by ^{31}P NMR	14
PC/PA	PA	PC	by ^{31}P NMR	14, 39
	--	--	by ^{31}P NMR, + Ca^{2+}	90
PC/SPH	PC	SPH	by ^{31}P NMR	14
PC/PG	PC	PG	by PIO & ^{31}P NMR	99
PC/PG	--	--	by PIO & ^{31}P NMR, + Mn^{2+} or Cd^{2+}	99
Vesicles by injection (VBI)				
PC/PE	--	--	by TNBS	131
	--	--	by TNBS, ± cyt b_5	118

[a]The information and design for this table were taken in part from Op den Kamp (135).

[b]VBS cases all reflect results of bath sonication of 1:1 mixtures of phospholipids in the absence of calcium or paramagnetic ions. References are to only the original report of the asymmetry, which may have been performed under slightly different conditions.

[c]Dashed entries reflect apparent symmetric distributions.

[d]Non-standard abbreviations are TNBS - titration with trinitrobenzene sulfonic acid, PIO - periodate oxidation, cyt b_5 - rabbit liver cytochrome b_5.

116, 106). Finally, the phospholipids in VBS are reported to respond to changes in external ionic environment in a manner very different than large diameter vesicles of the same composition (47). Table 3 notes these and other such findings.

The extent to which a unilamellar vesicle population is contaminated by multilamellar vesicles has been studied (11), and methods of centrifugation have been developed to separate homogeneous populations of large unilamellar from multilamellar vesicles (10, 111). Moreover, comparisons of the microscopic behavior and of many spectroscopic and calorimetric parameters of these two vesicle types have been made (cf. refs. 81, 138, 160). In general, differences between large diameter unilamellar vesicles and multilamellar vesicles of the same size are not as extreme as the differences observed between the large vesicles and VBS (cf. 160). The obvious exception is in the case of studies of membrane asymmetry, where contamination of unilammelar vesicles by multilamellar vesicles obscures the results of structural and dynamic measurements.

The noted differences in the observed physical properties of unilamellar vesicles of various diameters and between unilamellar and multilamellar vesicles highlight the need for a reliable method to produce unilammelar vesicles of defined diameter. Moreover, the diameter of vesicles to be used as a model system in the study of membrane asymmetry must be large enough to ensure that the packing constraints that control the distribution of phospholipids in VBS are absent. A variety of methods that have been described for the production of large unilamellar vesicles (cf. refs. 42, 181 for reviews). Included are ones based on the fusion of the smaller vesicles by sonication (163, 206), on the evaporation of organic solvent from co-sonicated aqueous dispersions of solvent, lipid and buffer (50, 179-181), on the effusion (boil-off) of an lipid-containing organic solvent into an aqueous solution at an appropriate combination of temperature and pressure (41, 42, 122), on the controlled dialysis of detergent from aqueous suspensions of de-

Table 3 Observed Effects of Curvature on Membrane Properties[a]

Effect Noted system components	Chemical Abstracts and Ref. Numbers
transbilayer asymmetry	CA09905034799, refs. 21, 131, 185, 186
lipid transfer cholesterol, phospholipids	CA10913106652, 10702012782, 10205041860, 10203019729
lipid-protein interactions cytochrome b_5, human apolipoprotein A-I, insulin, phospholipase A2	CA10419163975, 09719158376, 09715120889, 08823165973
membrane solute interaction pyrethroids, heptaene antibiotics, nonpolar molecules	CA10319155498, 10213105752, 09301002645
membrane elasticity	CA10313100432, 10119165875, 10103019375
phospholipid phase transition[b]	CA10301002453, 09309090634, 08809059584, 09113104043, ref. 101
fusion related events	CA10221181818, 10011081547, 09625212869, 09503020069
phospholipid packing[c]	CA09713105815, CA09309090634, CA09625212862, ref. 21
structural stability	ref. 116
permeability[d]	CA10221181818, ref. 101
optical properties	CA09317163801

[a]See also M. P. Sheetz and S. I. Chan (1972) *Biochemistry* 11, 4573.
[b]See also B. R. Lentz et al. (1976) *Biochemistry* 11, 4521, ibid., 4529 and J. Suurkuusk (1976) *Biochemistry* 15, 1393;
[c]See also C. Huang and J. T. Mason (1978) Proc. Nat. Acad. Sci. U.S.A. 75, 308 and A. Chrzeszczyk et al. (1977) *Biochim. Biophys. Acta* 470, 161.
[d]See also: Y. Barenholz (1979) *FEBS Lett.* 99, 210.

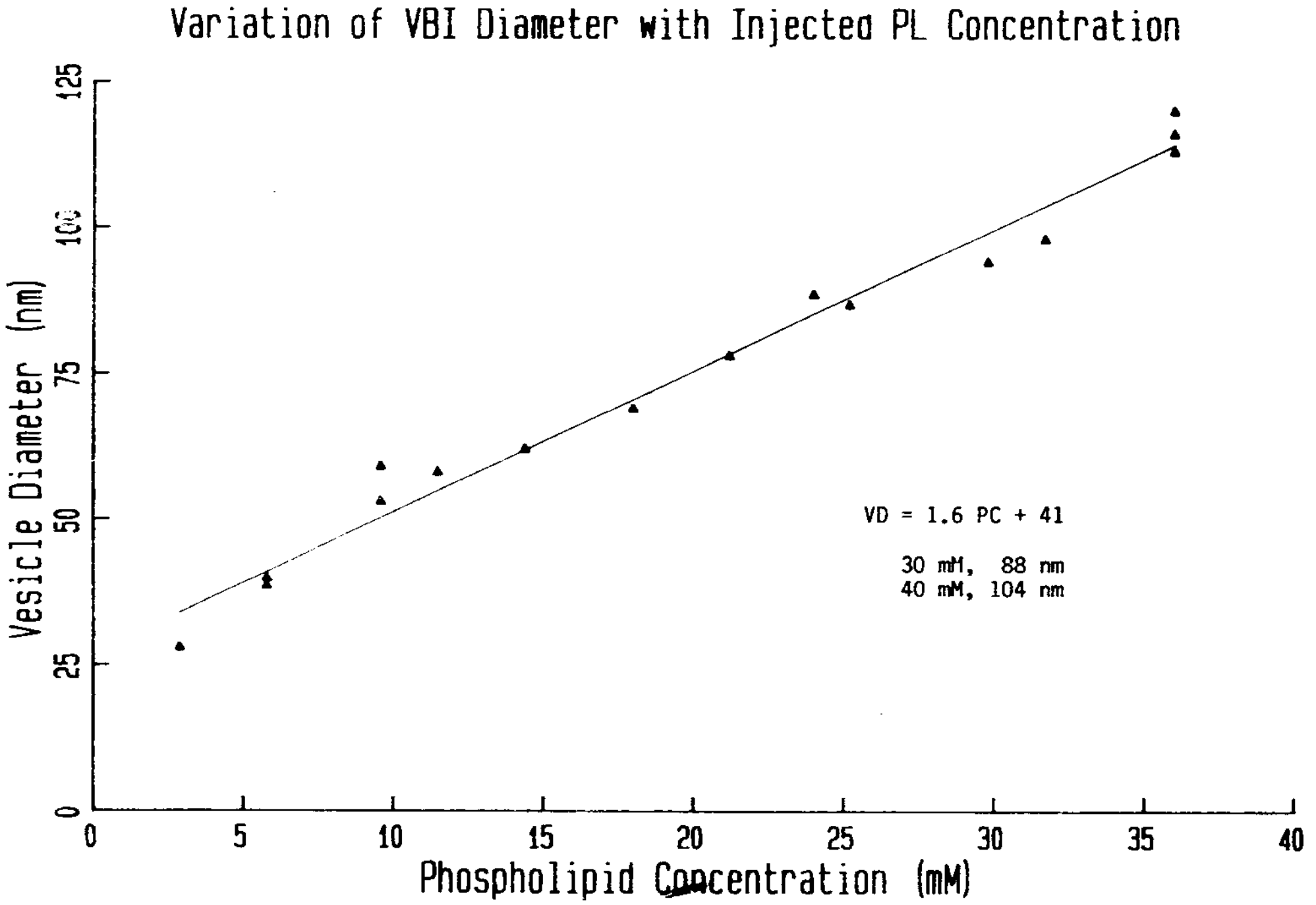
tergent, phospholipid and buffer (38, 89) and on the slow, controlled injection of a solution of phospholipid in ethanol into aqueous buffer (12, 103, 131, 181).

Each of these were evaluated as possible routine methods to produce large diameter vesicles. The method to produce vesicles by injection (VBI) was of special interest in that it had been shown to be useful for producing vesicles of a varying diameter, depending upon the concentration (in ethanol) of the injected phospholipid (Figure 2 and ref. 103).

As well, preparative methods were needed to separate unilamellar from contaminating multilamellar vesicles, and to isolate a population of vesicles that were fairly homogeneous in terms of diameter. The methods of centrifugation developed for the separation of VBS from multilammelar vesicles (10, 111) have been applied by Nordlund *et al.* (131) to the preparation of large diameter unilamellar vesicles. The authors note the need to determine actual conditions of centrifugation for each new preparation of vesicles studied, and to verify that multilamellar vesicles are indeed pelleted (10). The methods were rapid, simple and convenient, and were therefore evaluated and applied in the preparation of the large diameter vesicles used in this study.

The methods to separate phospholipid vesicles on the basis of effective diameter include those based on gel filtration chromatography, on centrifugation and on filter extrusion. Gel filtration methods using Sephadex CL-4B and 6B (82, 116, 181) were originally introduced by Huang for the preparation of VBS. This method was been extended to the separation of large diameter vesicles through the use of chromatographic materials with larger pore sizes, most notably Sephacryl S1000 (134, 151, 153, 187). Most common methods of preparative ultracentrifugation are generally not usefully applied to pure phospholipid vesicles, though a specialized, continuous-

Figure 2 Variation of VBI Diameter with Injected Phospholipid Concentration, from Kremer et al. (103). When DMPC in absolute ethanol is slowly injected into neutral Tris buffer with stirring, a largely unilamellar population of vesicles is formed. Shown is a graph based on data from Kremer *et al.* illustrating the dependence of the outside diameter of vesicles produced on the concentration of injected DMPC in ethanol. Diameters are based on light scattering measurements. The relationship between vesicle diameter (VD) and the concentration of PC in ethanol (PC) based on linear regression is presented, and the predicted diameters of vesicles from injections of 30 and 40 mM PC are noted.


Variation of VBI Diameter with Injected PL Concentration
Vesicle Diameter (nm)
0
25
50
75
100
125
0
5
10
15
20
25
30
35
40
Phospholipid Concentration (mM)
VD = 1.6 PC + 41
30 mM, 88 nm
40 mM, 104 nm

flow centrifugation technique has been successfuly developed (97). Filter extrusion methods using polycarbonate filters of well defined pore size have also been successfully used to prepare vesicles homogeneous with respect to diameter (cf. 50, 122).

In addition to these preparative methods, analytical methods were necessary to assess the homogeneity of the final vesicle preparation with respect to number of lamellae and to diameter. The review by Szoka and Paphadjopoulas (181) contains a helpful discussion of this topic. The methods for accurately assessing the average number of lamellae per vesicle in a preparation are the essentially the same methods used for determining the ratio of each phospholipid in the inner and outer leaflets of a vesicle, and are described in a section below. Analytical methods to characterize vesicles with respect to size include high resolution Fourier transform nuclear magnetic resonance techniques (NMR, described below) and methods of gel filtration chromatography, analytical ultracentrifugation, microscopy and light scattering.

The application of the low pressure gel filtration chromatographic technique described above to the analysis of vesicle size involves comparison of the elution characteristics of the vesicle population with those of standards of known diameter. The chromatographic materials used are the same as described above, and experimental conditions (column dimensions and flow rates) are chosen for higher resolution (4, 153). In addition to low pressure methods, high performance (pressure) liquid chromatographic materials for the same purpose have been introduced and evaluated (134). The application and features of the analytical utracentrifugation method of Huang *et al.* (cf. 82) are critically reviewed by Szoka and Papahadjopoulos (181), as are the uses of light and electron microscopic techniques. Light scattering measurements include classical techniques (181), and quasi-elastic laser light scattering techniques which estimate diameter from measurements of the time-dependent fluctuation of the intensity of scattered light (cf. 64).

Of these methods for fractionation and analytical characterization of vesicles, the chromatographic techniques are most accessible, and were therefore evaluated and subsequently used for the preparation of populations of large diameter vesicles homogeneous with respect to diameter and number of lamellae. Specifically, the Sephacryl S1000 technique developed by Reynolds, Tanford and co-workers (153, 187) and the HPLC technique introduced by Ollivon *et al.* (134) were evaluated. Filter extrusion techniques were used in a limited number of experiments (50). Magnetic resonance methods were evaluated and chosen (for reasons explained below) for analysis of the average number of lamellae per vesicle for populations of the large diameter vesicles.

2. Methods for Inducing Phospholipid Asymmetry

A number of methods have been developed to induce asymmetric distributions of phospholipid in biological and model membranes; complete descriptions can be found in the general reviews of Op den Kamp (135) and Thompson (185). As was noted above, compositional asymmetries resulting from the very small radius of curvature of vesicles by sonication provide one limited system for the study of membrane asymmetry. In addition, conditions have been elaborated where the apparent spontaneous transfer of phospholipid takes place between membranes of differing composition (cf. ref. 195). The most important methods, however, make use of either mammalian phospholipid exchange proteins, or phospholipid hydrolytic enzymes.

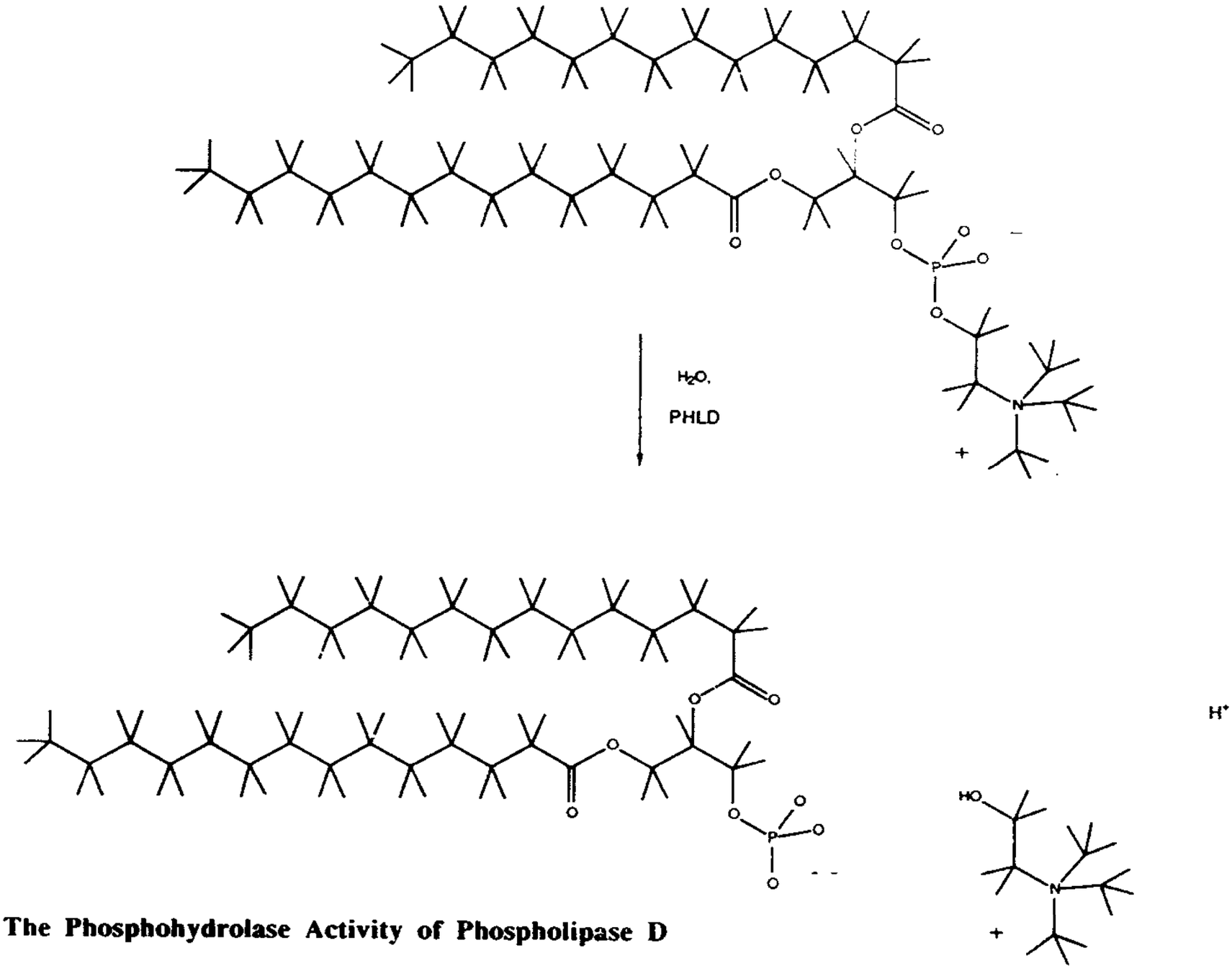
Phospholipid exchange proteins have been purified from a number of mammalian sources, including bovine liver and heart cytosol. [See *Chem. Phys. Lipids* 38 (1,2) 1985 for two complete issues devoted to studies of the purification, use and mechanism of a number of exchange proteins.] The role of these proteins *in vivo* is not yet clear; *in vitro* studies have demonstrated that the proteins can catalyze the efficient exchange of a variety of phospholipid species between the exposed leaflets of cells and vesicles (cf. ref. 77).

Specificity varies: Morrot *et al.* (127) report the use of a non-specific protein capable of the exchange of PC, PE, phosphatidylserine and their N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-labeled fluorescent analogs between vesicle and erythrocyte membranes. More common is the PC-specific bovine liver protein introduced by Zilversmit and co-workers (88) and further developed in the laboratory of T.E. Thompson (cf. 185). The PC-specific protein has, for example, been used to generate an asymmetry in isotopically labeled vesicles by the exchange of outer leaflet PC with PC from an unlabeled source. In this way, Shaw *et al.* (168) were able to determine that the halflife for the process of transbilayer redistribution (flip-flop) of PC was on the order of 31 days in vesicles prepared by sonication.

The phospholipid hydrolytic enzymes, or phospholipases, were introduced as tools early in the study of biological membranes, as Van Deenen *et al.* (189) note in their historical perspective on the subject. Earliest applications probed the effect of enzymatic hydrolysis on the function and morphology of whole cells. An early report from the laboratory Zwaal and co-workers (cited by Etemadi, ref. 55) noted that the reaction of phosphatidylcholine phosphohydrolase (phospholipase D, Figure 3) from cabbage was ineffective in hydolyzing PC to phosphatidic acid (PA) in the outer leaflet of erythrocyte membranes. Fuji and Tamura (61), on the other hand, noted that treatment with the same phospholipase resulted in increasing invagination of the erythrocyte membrane with increasing hydrolysis of PC to PA. Treatment with both phospholipase C (yielding phosphoryl-choline and diacylglycerol) and phospholipase D were shown to inhibit phospholipase A2-induced membrane crenation.

Recent work has in part continued to focus on the consequence of the action of phospholipases on biological membranes. Phillipson and Nishimoto (139), for instance, note that Na^+/Ca^{2+} exchange is stimulated in cardiac sarcolemmel vesicles after phospholipase D treatment. Clancy and

Figure 3 The Phosphohydrolase Activity of Phospholipase D. Dimyristoyl PC is shown as substrate and dimyristoyl PA and choline as products of the reaction catalyzed by the phosphohydrolase of activity of phospholipase D. Carbon and hydrogen atoms are represented by the intersections of four bonds, and by the unlabeled termini of bonds, respectively. All other atoms are labeled. The presentation of bond angles roughly reflects those of the extended form of crystalline DMPC (210). Note that the conversion of the zwitterionic substrate results in the production a proton and one equivalent each of anionic phospholipid and free choline. The reaction is a special case of the more general alcohol transferase reaction: exchange of the choline moiety of PC for a variety of alcohols is also catalyzed by the cabbage enzyme.



The Phosphohydrolase Activity of Phospholipase D

coworkers (31, 32) observed effects on the K_m of β-D-hydroxybutyrate dehydrogenase after treatment of submitochodrial membranes with a purified preparation of cabbage phospholipase D. Research on phospholipase C has burgeoned with the discovery (see 62 and refs. therein) that products of a phosphatidylinositol-dependent phospholipase C serve as a cellular "second-messengers."

Recent mechanistic work on the phospholipases has focused on enzyme structure-function questions (e.g., the work of R.L. Heinrickson, F. Kezdy, P.B. Sigler, M.-D. Tsai and others), on the characteristics of their "activation" (62) and on the use of model membranes as their substrates. Model membranes as sources of PC substrate have been used with phospholipase D (PHLD), in particular, for studies of membrane asymmetry.[1] Described in some detail below are reports of PHLD experiments that were designed to (i) understand the role of membrane surface charge in physiologic processes, (ii) determine the kinetics of the enzyme-catalyzed reaction with a bilayer-form of substrate and (iii) probe changes in membrane structure following an asymmetric perturbation to the bilayer.

Early attempts at more careful mechanistic and physical measurements were hampered by "the lack of readily available pure [PHLD]" (55). The work of Allgyer and Wells (5) and of Clancy and co-workers (31, 32) resulted in methods to separate the cabbage PHLD from the contaminating activities responsible for earlier artifacts. In a recent model membrane study, Wissenberg (204) reports efforts in this laboratory to extend the work of Clancy to the hydrolysis of vesicles

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[1]Of the phospholipases, only phospholipase D (reviewed in refs. 76, 90 and 183) converts one phospholipid species (PC) to another phospholipid species (PA). Its action is the most gentle in terms of maintaining the integrity of the bilayer, and it is therefore of most use in terms of studies of phospholipid asymmetry. The neutral lipid and lysophosphatide products of phospholipases C and A2 are known to be disruptive to bilayer membrane phases. Interest in the enzyme has been stimulated by its cloning from a variety of sources, and by implication of its activity in the process of seed germination.

prepared by sonication. In these preliminary experiments, egg yolk PC or DMPC vesicles were prepared and stored for periods of up to one week. These preparations were then used to compare the rate of hydrolysis of vesicle PC with the rate observed for PC dispersed between aqueous and organic phases in a two phase system. Differences were noted, consistent with reported variations of PHLD activity with form of substrate (see Table 4 and ref. 40).

Moreover, Wissenberg found that hydrolysis of egg yolk PC in vesicles prepared by sonication reached a plateau after ≈30 mol% of the total vesicle phospholipid, corresponding to about half of the outer leaflet phospholipid, was converted to PA. Under similar conditions, DMPC vesicles yielded less that 5% conversion to DMPA. Reported reactions were stopped under conditions that would disrupt the membrane. Moreover, characterizations of reaction products for byproducts or oxidative impurities, and of product "vesicles" in terms of the barrier properties of the membrane, the number of lamellae per vesicle, the distribution of PC and PA across the bilayer, etc., were beyond the scope of the investigation.

In the only recent report of a kinetic analysis of PHLD with a vesicle substrate, Nakagaki *et al.* (128)[1] present data for the PHLD-catalyzed hydrolysis of vesicles produced by co-sonication of DMPC and cholesterol. Reactions were followed by the increase in the fluorescence of 8-anilino-1-naphthalene sulfonate, apparently reporting the increase of PA in the membrane. The authors report that the maximum rate of hydrolysis at room temperature is observed at pH 5.6 with a Ca^{2+} concentration of 60 mM and a membrane cholesterol content of 31% (w/w). At least two distinct rates are noted during the time-course; the existence of two distinct populations of substrate PC are inferred, suggesting the possibility of migration of inner leaflet PC to the exposed vesicle surface.

[1]At least one similar, subsequent report has appeared in *Yakagaku Zasshi*. It originates in the same laboratory as ref. 128.

Table 4 Reported pH Optima for PHLD-catalyzed Hydrolysis of Phosphatidylcholine in the Presence of Calcium.[a]

System	Substrate	Optimal pH	$[Ca^{2+}]$ (mM)	Ca^{2+} / PC (mol/mol)	Ref.
ether/water biphase	egg yolk PC	5.6	40	40	40
	egg yolk PC	5.6	100	15	35
	egg yolk PC	5.6	100	50	25
solution	dihexanoyl PC	7.25 > 6.25	50	25	5
	dihexanoyl PC	6.25 > 7.25	2	0.5	5
monolayer	unspecified PC	6.4	0.2	--	94

[a]All optima reported are for reactions catalyzed by partially purified preparations of phospholipase D from cabbage in buffered solutions at approximately equal ionic strengths. Substrate concentration varied somewhat, so both absolute concentration of Ca^{2+} and the ratio of Ca^{2+} to substrate are reported.

In the only report of an attempt to use PHLD to generate asymmetric vesicles for further physical study, DeKruiff and Baken (43) present data on the apparent transbilayer redistribution (flip-flop) of PA following PHLD-catalyzed hydrolysis by enzyme purified from cabbage according to the method of Dawson and Hemington (40). The authors briefly address the problem of the interaction of Ca^{2+}, theretofore generally considered necessary for the hydrolytic reaction, with the product PA, and the phenomena of phase separation, vesicle aggregation and fusion, and transbilayer redistribution of PA that result from the interaction. DeKruiff and Backen concur with the few previous investigators (Table 5) that have reported that the phosphohydrolase reaction will proceed in the absence of calcium.

In the study, the distinct phosphorus-31 nuclear magnetic resonance (^{31}P NMR) signals of PC and PA were used to quantitate each phospholipid. A series of carefully controlled experiments using the line-broadening reagent Cd^{2+} were performed to distinguish outer and inner leaflet resonances and consequently to determine the stability of the asymmetric distribution of PA. DeKruiff and Backen report a very brief (30-40 min.) halflife for PA in the outer leaflet of the product PC/PA vesicles. The mechanism of rapid translocation in this study is perhaps indicated by the recent report of the Cd^{2+} catalyzed translocation of acidic phospholipids in the same type of vesicles (99). Accordingly, the X-ray crystallographic phases of Ca·PA and Cd·PA are reported to be comparable, unlike those for Ca·PA and other divalent cation·PA complexes (73, 115, 125).

The extensive literature on phospholipase D (cf. 76), and the experience of this laboratory in its purification from cabbage (31, 32, 204) encouraged its selection as a means to induce an asymmetric distribution of phospholipid in a model membrane system, specifically a PC/PA asymmetry in well-characterized large diameter unilammelar vesicles. An added incentive was presence of the more general alcohol transferase activity with the hydrolase activity of the enzyme

Table 5 Reported Relative Rates of PHLD-catalyzed Hydrolysis of Phosphatidylcholine in the Absence of Calcium.[a]

Assay System Substrate	Enzyme Preparation	% Maximal Rate[b]	Ref.
ether/water biphase			
egg yolk PC	crude plastid	100	144
soya PL	crude plastid	63	38
egg yolk PC	acetone ppt	0	207
egg yolk PC	from chl-rich outer leaves	52, 20, 40	40
egg yolk PC	from chl-poor inner leaves	19, 16, 12	40
egg yolk PC	from chl-free heart	27, 14, 12	40
Vesicles by sonication			
egg yolk PC	from chl-poor inner leaves, according to ref. 40	60	39

[a]All rates reported are for reactions catalyzed by preparations of phospholipase D purified from cabbage to the extent indicated. Assays were performed in buffered solutions at Ca^{2+} concentrations of 40-100 mM. Ether biphase assays were comparable to those of Clancy (31). For conditions of VBS reaction, see ref. 43. Non-standard abbreviations are PL - phospholipid, and chl-chlorophyll.

[b]Following rates are for crude, 55°C supernatent, and 55°C precipitate, respectively.

II. Materials

A. General

All glassware was detergent-cleaned, rinsed thoroughly with glass distilled water and air-dried. Glassware exposed to organic solvents was rinsed with a small volume of the solvent and redried prior to use. In experiments where it was necessary to remove metal impurities, washed items of glass and poly(tetrafluoroethylene) were prepared for use by exposure to aquaregia (18% nitric acid [v/v] in concentrated HCl, stored in an opaque, tightly-stoppered bottle); items were were then rinsed repeatedly with distilled water and air dried. Water was deionized, then distilled and collected in glass. All acids and bases (except those containing stable isotopes, see below) were BakerAnalyzed grade from J. T. Baker. Nitrogen, helium and argon were dry-grade from Linde.

HEPES and TES were Ultrol grade from Calbiochem-Behring. MES, PIPES and other Good buffers (57, 66) were from Research Organics (Cleveland, OH). Histidine, Tris HCl, Tricine, succinate, *myo*-inositol and dithiothreitol were from Sigma Chemical (St.Louis, MO). Praesedymium chloride was from Apache Chemical (Seward, IL) and was processed as described below. Ytterbium chloride hexahydrate was from Hudson Laboratories (99.999%, Hudson, FL). Phosphorous acid, trimethyl ester (trimethoxyphosphite) from Wilmad (Buena,NJ). All other inorganics were BakerAnalyzed from J.T. Baker.

B. Phospholipids and Related Compounds

DMPC, hydrogenated egg yolk PC and egg yolk PC were most often purchased from Avanti Polar Lipids (Birmingham, AL). For some experiments, egg yolk PC was the purest available product from Sigma Chemical, or was isolated from its natural source (174) and chromatographed under inert conditions as described by Gilmore *et al.* (63). See below for sources of stable and radioisotope-labeled PC. Dihexanoyl PC, a kind gift of Dr. John Cronan, Department of Microbiology, was from Calbiochem-Behring. The sources of PA were primarily as described above for PC. In some cases, egg yolk PA was produced from isolated PC by phospholipase D catalyzed hydrolysis (35) and subsequently chromatographed as noted for PC above. When PA was supplied as a sodium salt, it was converted to the free acid by an HCl wash procedure (see below) communicated by Dr. Walter Shaw of Avanti Polar Lipids.

DL-α-Glycerophosphate hexahydrate and 1,2-Dimyristoyl glycerol were from Serdary Research Labs (London, Ontario). Phosphorylcholine chloride, calcium salt (98%) and choline chloride (Gold Label) were from Aldrich Chemical. L-α-phosphorylethanolamine was from Sigma.

C. Organic Solvents

Ethylene Glycol was from Malinckrodt. Pentane and hexane were Nanograde and low-boiling petroleum ether, and diethyl and diisopropyl ethers were AnalyticalReagent grade from Malinckrodt. Solvents used in characterizing spectral shifts of laurodan were Aldrich Gold Label, except for absolute ethanol (from U.S. Industrial Chemical, Tuscola, IL) and water (described above). All other solvents were BakerAnalyzed, spectrophotometric grade from J.T. Baker. The sodium salt of dodecylsulfate (SDS) and acrylamide were electrophesis grade from Bio-Rad.

D. Fluorescent Probes and Isotopes

N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) phosphatidylethanolamine (N-NBD-PE), was purchased from Avanti Polar Lipids. 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-[4-(trimethylammonio)-phenyl]-6-diphenyl-1,3,5-hexatriene (TMA-DPH) were from Molecular Probes (Eugene, OR). The prodan analog 6-propionyl-2-dimethylnaphthalene (laurodan) was the kind gift of Drs. Gerard Merriott and Gregorio Weber. It was synthesized in the Weber laboratory by Ms. F. Farris, according to their published method (200).

Deuterium oxide (99.8 mol%) was from Bio-Rad. Isotopically pure deuterium chloride, deuterium hydroxide, deuterated methanol and phosphoric acid (85% in $^2H_20$) were purchased from Cambridge Isotope Laboratories (Cambridge MA). [Choline, N-$^{13}CH_3$] DMPC was a special order from Avanti Polar Lipids (Birmingham, AL).

[Choline, N-C^3H_3] DMPC (58.0 Ci/mmol, batch 1782-239) was purchased from New England Nuclear. A small quantity of [1-palmitoyl, 1-^{14}C] DPPC, (127.0 mCi/mmol, batch 1703-083, also from New England Nuclear) was the kind gift of Dr. Ana Jonas. [1,2-^{14}C] ethanol (batch 381, 1.09 mCi/ml), 98% pure by gas chromatography, was from Research Products International (Mount Prospect, IL) and was stored as a ten-fold dilution in absolute ethanol. Stock solutions of ^{14}C ethanol (3.6 µCi [8.1 x 10^6 dpm] / ml) was prepared for vesicle injection by dilution.

E. Chromatography

Minimum dead-volume chromatographic columns [0.9 cm (i.d.) x 50 and 100 cm, and other standard sizes] and their threaded-end caps were machined from commercially available plexiglass

tubing and block by machinists in the School of Chemical Sciences. Filter papers disks of standard sizes were Whatman #1 from Whatman Paper. γ-Aminopropane agarose was from the synthetic stock of Dr. Robert Clancy, was synthesized as described (31, 32; 0.3 μmol reactive amine / ml settled gel in both) or was from Sigma Chemical (#4893, lot 14F-9525, 5 μmol/ml). Sephadex G-25 (medium grade) and Sephacryl S1000 (superfine grade) were purchased from Pharmacia Fine Chemicals. Suspensions [2.5% (w/v)] of carboxylated latex microspheres (diameters: 70 ± 7, 100 ± 9 and 190 ± 10 nm) and of coumarin-labeled carboxylated latex microspheres (diameter: 80 ± 6 nm) were from Polysciences (Warrington, PA).

F. Sources of Enzyme

Some preparations of phospholipase D from cabbage were the purest available products from Sigma Chemical. Kilogram quantities of Savoy cabbage, the source of most preparations, was purchased from J.M. Jones or Dohme Produce (Urbana-Champaign, IL).

III. Methods

A. Phospholipid preparation

Phospholipids were judged to be free of impurity by one- and two-dimensional thin layer chromatography (TLC, ref. 156) and by absorption spectroscopy (99). Phospholipid concentrations of solutions were determined as inorganic phosphate after dry ashing according to the method of Kates (93), or as intact phospholipid according to the method of Stewart (177). Volumes of stock solutions containing desired amounts of phospholipid were measured and transferred by Hamilton Microliter syringe. References made to *the standard method for preparing a dry sample of phospholipid* refer to the following. Dissolving solvents were removed from phospholipid aliquots first under a stream of nitrogen at 37°C, then *in vacuo* overnight. The vacuum dessicator was vented with dry nitrogen or argon. Dry phospholipid was stored under such an atmosphere at 4°C in the dark until use.

The sodium salt of PA was converted to the free acid by the following procedure (Dr. Walter Shaw, Avanti Polar Lipids). PA was dissolved in argon-bubbled $CHCl_3/CH_3OH$ (2:1) at a concentration of 30 µg/ml. 0.2 Volumes of 0.001 N HCl was added, and here in subsequent washings the vessel was purged with argon or nitrogen, sealed and mixed for 2 min. The phases were allowed to separate and the aqueous phase was removed. The organic phase was washed

three times with 0.2 vol CH_3OH/H_2O (1:1), each time with care being taken to exclude oxygen, and then taken to dryness under vacuum or nitrogen. The free acid of PA was dissolved in argon-purged hexane/ethanol (98:2, v/v) and stored under the conditions described above for PC.

Radiolabeled preparations of phospholipid were prepared by the addition of [choline, N-C^3H_3] DMPC to solutions of DMPC and egg yolk PC of known concentration (see below). Volumes of high specific activity DMPC were measured and transferred by glass microcap (VWR/Drummond). Final activities were determined by scintilation counting and were approximately 450 μCi (1×10^9 dpm)/mmol in the enzyme assay preparation and 9 μCi (2×10^7 dpm) / mmol in column chromatographic preparation, reflecting final concentrations of labeled DMPC in unlabeled PC of no greater than 0.001 mol%. For some hydrolysis experiments, ^{14}C-PC was measured and transferred to label a portion of the enzyme hydrolysis stock of ^3H-PC. The final activity of this double-labeled preparation was 450 μCi (1×10^9 dpm) ^3H-DMPC and 408 μCi (9×10^8 dpm) ^{14}C-DPPC per mmol egg yolk PC. The final concentration of radiolabel (^3H and ^{14}C synthetic phospholipid) in egg yolk PC was 0.36 mol%.

Fluorescently labeled preparations of phospholipid for HPLC experiments were prepared by the addition of N-NBD-PE to DMPC and egg yolk PC. Final concentration of N-NBD-PE in PC was 0.5 mol%. DPH and TMA-DPH labeled phospholipid preparations were prepared as indicated in the figure legend for each experiment.

B. Measurement of Radioactivity

Measurements of radioactivity were performed with a Beckman LS-7000 Scintillation Counter with preset descriminators for ^3H and (^{14}C - ^3H). Quench corrections were performed and counting

efficiences were determined according to the directions of the manufacturer, using the H# feature of the instrument. Efficiencies for ^{3}H and ^{14}Cwere generally approximately 30% and 90%, respectively.

Samples for counting were prepared in suitably sized glass vials using the xylene/triton water-in-oil emulsion scintillator of Anderson and McClure (6) with added optional 0.02% 1,4-bis [2-(5-phenoxazolyl)] benzene. Typically, the combined volume of sample and added water was such that the ratio of aqueous to scintillator was held at 0.14 (12%, v/v). When an experimental protocol required counting different aqueous volumes, or equal volumes of different pH or ionic strength, adjustments were made so that counting conditions were consistent within each set of samples whose counts were to be compared.

Rotary mixing was used to insure that samples were opalescent or clear at the time of counting. When the stability of the emulsion allowed, final measurements were made after storing samples for 5 hrs. in the dark. Background was determined for each set of samples by measuring the scintillation of a matching aliquot of radioactivity-free aqueous in scintillator.

Prior to performing experiments requiring the measurement of absolute levels of ^{3}H and ^{14}C in the same sample, matching samples were prepared without the ^{3}H label. The radioactivity of the ^{14}C label reported in the ^{3}H and (^{14}C - ^{3}H) channels was used to determine the ^{14}C channels ratio, $^{3}H/(^{14}C - ^{3}H)$, a constant for a given set of counting conditions. In the double-label experiment, the radioactivity reported in the (^{14}C - ^{3}H) channel for each sample was multiplied by this constant to determine the amount of radioactivity in the ^{3}H channel attributable to ^{14}C. This amount was substracted from the radioactivity reported in the ^{3}H channel, and added to the radioactivity reported in the (^{14}C - ^{3}H) channel, to determine the actual values for ^{3}H and ^{14}C, respectively.

C. Vesicle preparations

Vesicles by dispersion (DISP) of phospholipid in buffer were prepared by a method based on the principle introduced by Bangham *et al.* (9). Appropriately measured volumes of phospholipid stock solution were transferred into clean, solvent-rinsed glass snap-neck ampules (Kimble). The ampules were then stored with the precautions described above for phospholipid storage. On the day prior to use, phospholipid in the ampule was taken to dryness according to the general method.

On the day of use, a volume of buffer was warmed to a temperature approximately 10°C greater than the T_c of the highest melting phospholipid component of the experimental sample. (Hereafter, the temperature corresponding to 10°C greater than the T_c of the highest melting phospholipid will be referred to as "T_c+10".) The ampule was removed from the desicator, measured buffer was quickly added, and the ampule was then flushed with dry nitrogen and flame sealed. The phospholipid was dispersed at the same temperature by rotary mixing (Scientific Products #58220) at maximum speed for 3 min. A second 3-min. mixing was performed if undispersed phospholipid was apparent on the inside surface of the ampule. Dispersions were incubated for 30 min at T_c+10°C prior to experimental use.

References to *the standard method for producing DISP* indicate preparation from 40 μmol phospholipid and 4 ml buffer, for a 10 mM final concentration. 5 ml ampules were used for these preparations. When egg yolk phospholipids were used, the "T_c+10°C" was typically 25°C.

Vesicles by sonication (VBS) were produced by a method similar to that of Barrow and Lentz (11), who report optimal conditions for substituting a cup-horn device for the well characterized

micro-tip sonicator of Huang (82). In this method, a flame-sealed ampule of DISP was centered approximately 5 mm above the horn of a Bronson 10-375 Sonicator so that the base of the ampule was positioned in the cavitation umbrella (11) when the unit was powered. Samples were sonicated in continuous mode at 100% power, for 30 min. longer than the time required to reach minimum optical density at 300 nm. Temperature was held constant at T_c+10°C by the flow of a Lauda K-2/R continuously circulating water bath.

References to *the standard method for producing VBS* indicate preparation from the 10 mM standard preparation of DISP described above. Duration of sonication was typically 90 and 120 min. for egg and DM phospholipids, respectively.

Vesicles by fusion were produced by a method based on the method developed by Schullery *et al.* (163) as described by Wong *et al.* (206). VBS of DMPC were produced as described above using the phospholipid and buffer concentrations prescribed, except that MES was substituted for PIPES, and pH was adjusted to 6.0. A homogenous population of vesicles was produced from this preparation by centrifugation as described below. Fusion of vesicles was initiated by cooling to and incubating at 4°C.

Vesicles by evaporation were produced by an adaptation of the "reverse phase" evaporation method introduced and developed by Szoka, Papahadjopoulos and others (179, 180, 50). Here, DMPC vesicles by evaporation were prepared as described by Szoka, *et al.* (179), except at 10 mM concentration and at one-third scale using diisopropyl ether-chloroform (1:1) as solvent and 2 mM histidine, 100 mM NaCl, 0.1 mM EDTA pH 7.4 as encapsulation buffer (181). The evaporation vessel was a custom-blown thin-walled 5 ml roundbottom flask with screw top closure. Sonication was as described in the above section on VBS, except shorter in duration (179). Rotary evaporation was performed on a Brinkman/Buchi Rotovap-R at approximately 200 rpm (180),

using suitable adaptors (Kontes Glass, Vineland, NJ) to join the threaded and ground glass tapered openings. Vacuum (179) was provided by a Cenco #91140 pump; vacuum levels were monitored using a Marshaltown gauge (#90532) and were adjusted by varying nitrogen flow from a supply cylinder using a metering stopcock (Ace Glass #8195). All other conditions were as described (179), except that the procedure was evaluated without added extrusion steps.

Vesicles by effusion were produced by the method of Deamer and Bangham (41), in consideration of later refinements and suggestions (122, 42). DMPC or egg yolk PC (2 mM in petroleum ether, ref. 122, or pentane, ref. 41) were effused under the conditions and using the apparatus described for pentane effusion except that a second, cold condensor surmounted the effusion condensor, compression of the syringe plunge was performed manually and a 30 ml volume was effused into a 1:10 dilution of the phosphate-buffered saline (122) described above. Alternatively, vacuum effusion at lower temperatures was attempted (41). In either case, vesicles were extruded as described by Deamer and Bangham, and concentrated by the general described below.

Vesicles by detergent dialysis were routinely prepared by Mr. Joseph Cimino using the β-D-octyl glucoside version of the method introduced by Kagawa and Racker (89). Details of his application of this method to a variety of phospholipid and protein systems are available (38).

Vesicles by injection (VBI) were routinely prepared by the method introduced by Batzri and Korn (12) and subsequently developed by other laboratories (103, 131). Briefly, individual phospholipids or phospholipid mixtures were dispensed into a suitable glass container and taken to dryness according to the general method. Samples were redissolved in absolute ethanol, with care being taken to accurately measure volume. Concentrations of phospholipid in ethanol were dictated by desired vesicle size (103) and were generally 30 or 40 mM (see Fig. 1). The volume of

injected ethanol was determined by the final desired vesicle volume and by the general requirement of a post-injection concentration of ethanol in buffer of 5% (v/v). In NMR and other experiments requiring vesicle phospholipid concentrations significantly greater than 1.5 mM, a post-injection concentration of ethanol in buffer of 25% (v/v) was used as suggested by Nordlund *et al.* (131).

Ethanol solutions were taken up in 1 or 5 ml Hamilton Gastight syringes (#1001 or 1005, respectively) through flexible 20 gauge poly(tetrafluoroethylene) needles (Aldrich Chemical) tipped by a 8 cm length of custom blunt-cut 18 gauge stainless steel tubing. The desired volume of encapsulation buffer was transferred to the injection vessel, typically a small flat-bottomed glass vial, a poly(tetrafluoroethylene) coated stirbar was added, and the vessel was purged with nitrogen and cappedwith a syringe septum. The stainless steel tubing was passed through the septum and immersed to approximately half the depth of the encapsulation buffer.

The solution was rapidly stirred by magnetic stirring motor; rate of stirring that was held constant from experiment to experiment. Injections were performed at T_c+10°C. When necessary, a specially constructed vial and water jacket assembly was used, and temperature was maintained by the continuous flow of a Lauda K-2/r circulating water bath.

Slow compression of the syringe plunger was accomplished using a Sage Instruments model 355 syringe pump kindly on loan from the laboratory of Dr. Timothy Nieman, Department of Chemistry. It was set at 4% flow in its 1/10 range and at 8% in its 1/100 range for injections with 1 and 5 ml syringes, respectively. Under these conditions, rates of injection were reproducibly in the range of 300-400 µl/hour.

At times, this volume was diluted four- to ten-fold with encapsulation buffer, and vacuum dialyzed as described below. More generally, the entire volume of VBI in buffer/ethanol was applied to a column of Sephadex G25 for separation of VBI from ethanol (131). Sephadex was swollen and packed according to the directions of the manufacturer. Samples with post-injection volumes of 2 ml or less were chromatographed on 20 ml gel in 0.9 cm x 35 cm column, with volumes of 2-4 ml on 40 ml gel in a 1.5 cm x 30 cm column and with volumes of 4-8 ml on 80 ml gel in a 2.5 cm x 20 cm column, all equilibrated with encapsulation or other isotonic buffer.

Samples were typically applied to a suitably-sized filter paper disk placed on the drained gel surface. A flow rate of approximately 3 ml/min was maintained by gravity flow and by metering out-flow from the column. Collection of eluted fractions began with sample loading, and was performed by a Gilson Micro-Fractionator. Typically, fraction volumes were 25% of applied sample volume. Elution profiles were determined by measurement of optical density at 300 nm, or by scintillation counting of aliquots from fractions. The separation performed on samples prepared with [1,2-^{14}C] ethanol was as described except that all manipulations were performed in a fume hood, and fractions were sealed immediately after advancement of the fraction collector.

References to *the standard method for producing VBI* indicate a preparation made by injection of 0.1 ml 30 mM egg yolk or DM PC in ethanol by 1 ml syringe into 1.9 ml of encapsulation buffer, followed by chromatography on the 20 ml Sephadex G25 column as described.

D. Concentration and fractionation of vesicles

In all early VBI experiments, and in later experiments requiring greater than standard vesicle phospholipid concentrations, vesicles were simultaneously dialyzed and concentrated using the -p–micro-ProDiCon vacuum dialysis system and PA-10 (10 kD cut-off) dialysis membranes from

Biomolecular Dynamics (Beaverton, OR). When the purpose was dialysis as well as concentration, samples were first diluted to 20 ml. Dialysis was against two subsequent 1 liter volumes of encapsulation or other isotonic buffer, each for 10 hr.

After the initial 10 hr. dialysis, the sample was diluted to 10 ml with mixing of the cone volume. Poly(tetrafluoroethylene) inserts used were such that the final volume was typically 0.1 ml. After the second 10 hr. dialysis, this 0.1 ml or other final volume was diluted to half of the final desired volume and removed. The unit was then washed with aliquots of dialysis buffer such that the combined volumes of transferred sample and washes equalled the final desired vesicle volume. Vesicle phospholipid concentration was determined by scintillation counting, or chemically as described above.

An homogenous population of VBS was routinely produced by the high-speed centrifugation technique introduced by Barenholtz, *et al.* (10) and modified in accordance with the observation of Lentz, *et al.* (111). Conditions were uniformly 159,000 x*g* for 60 min. and were performed at T_c+10°C in a Beckman L-8 Ultracentrifuge, in rotors and appropriately sized polycarbonate tubes from the same manufacturer. The duration of 60 min. excluded the period of acceleration. Specific conditions deemed equivalent by calculation according to Beckman product literature were 48,000 rpm in a type 75 Ti rotor, 46,000 rpm in a type 70.1 and 48,500 rpm in a type 50 Ti. Vesicles from region III, the cylindrical volume above the uppermost edge of pelleted dispersions, were considered to be homogenous (10).

In selected experiments, the ethanol-free VBI fraction from Sephadex G25 was centrifuged as suggested by Nordlund, *et al.* (131) to produce an homogenous population of VBI. These centrifugations were as described above, except that conditions were 96,500 x*g* for 60 min., with corresponding changes in velocity for each rotor. Alternatively (cf. ref. 50), this VBI fraction was

extruded through a Nucleopore polycarbonate membrane with 0.2 um pore diameter (Nucleopore, Pleasanton, CA), followed by centrifugation at 10,000 x*g* for 20 min. in the manner described above. In some experiments the final 20 min. centrifugation was omitted. These exceptions are noted in the figure legends.

E. Analytical Gel Filtration Chromatography of Vesicles

Columns of Sephacryl S1000 were packed for use in determining ranges of vesicle diameters (153) according to a method adapted from the directions of the manufacturer (Pharmacia). A volume equal to 160% of the desired total column volume, V_t, was measured from the commercially available (85%) suspension in water. The gel was allowed to settle, water was decanted, and 40 mM PIPES, 40 mM NaCl, 0.04% NaN_3 pH 6.8 was added to produce a mixture that was 70% settled gel. This mixture was degassed on medium vacuum overnight and packed in 0.9 cm x 50 cm or 100 cm columns fitted with extension reservoirs.

The 50 cm column was packed by gravity flow at approximately 18ml/hr until the extension reservoir was empty of gel, and by the downward flow of a Gilson Minipuls 2 peristaltic pump at approximately 24 ml/hr until the gel bed ceased settling. Flow rates for the 100 cm column were half those of the 50 cm column. After packing, columns were equilibrated by successive flows of column buffer (20 mM PIPES, 20 mM NaCl, 0.02% NaN_3 pH 6.8) for 8 hrs., and of SDS-column buffer (as above, with 2 mM SDS) for 24 hrs. The column was fitted with a flow adaptor, or the gel bed surface was overlaid with a porous filter-paper disk, and bed height was measured for calculation of SDS-column volume, V_t (unsaturated, +SDS).

Elution volumes of particles of known diameter were determined using commercially available preparations of carboxylated latex microspheres and coumarin-labeled carboxylated latex microspheres. Preparations were diluted 1:10 in SDS-column buffer, and 0.2 ml volumes were applied to the 50 cm column via the flow adapter, or by careful dropwise transfer onto tne paper disk above the drained gel surface. A flow rate of 24 ml/hr was maintained as described above. Collection of eluted fractions began with sample loading and was performed by Gilson Micro Fractionator; 19 drop (≈335 µl) fractions were collected. Elution profiles were determined for all (non-labeled) or visibly fluorescent (coumarin-labeled) carboxylated latex microspheres fractions by absorbance measurements on the spectrophotometer described below; wavelengths were 300 nm and 460 nm, respectively. Alternatively, fractions for remeasurement were indicated from absorbance profiles generated by an Altex 153 flow-through absorbance measurement system with a linear Instruments 161 Chart Recorder, kindly on loan from Dr. John Clark. Comparable experiments were performed on the 100 cm column at approximately half the flow rate and twice the sample size of the 50 cm column. Both columns were subsequently washed free of SDS and reequilibrated with column buffer at experimental flow rates for 24 hrs.

S1000 columns were "saturated" by the recycled flow of a 40 ml volume of 10 mM PC VBS (153). Here, VBS were produced by pulsed sonication of a 50 ml volume of 15 mM DISP of hydrogenated egg yolk PC in 20 mM PIPES, 20 mM NaCl, 10 mM EDTA, 0.02% NaN_3 pH 6.8 (column buffer + EDTA). The probe was immersed to half the depth of the DISP in a 250 ml pyrex beaker, and sonicated on a BronsonW-350 Sonifier at 60% power and 40% duty cycle for 120 min. (K. Oden, personal communication). Temperature was maintained at approximately T_c+10°C by the addition of ice to the still waterbath surrounding the sample. Crude VBS were centrifuged at 30 000 x*g* (16 000 rpm in a DuPont/Sorvall SS-34 rotor) for 30min. at 25°C, and the region III supernatent (see above and ref. 10) was carefully removed for use as saturant.

This preparation of VBS was drawn and applied to columns by peristalic pump; recirculation was initiated with the appearanceof the turbid vesicle peak, and was continued for 8 hrs. Pump settings were maintained as above, although corresponding flowrates were 30-50% lower. Following saturation, the flow adaptor was rinsed or the paper disk replaced, and the column was washed for 12 hrs. with column buffer + EDTA. After reequilibration with the original column buffer for 4 hrs., the bed height was measured for calculation of saturated column volume, V_t, (saturated,-SDS).

Void volume (V_o) of Sephacryl S1000 columns were determined using samples of a heat-killed chromogenic microorganism, according to a method communicated by Ms. Shelley Weiss (Pharmacia). Here, a 0.5 ml aliquot of stationary phase *Seratia* (the gift of Dr. Charles Pratt, Department of Microbiology) was innoculated into 40 ml tryptone soybroth and incubated at 25°C for 4 hr. with shaking. The suspension of bacteria was heat-killed by incubation as 60°C for 2 hr, and bacteria were harvested by centrifugation at 8,500 x*g* for 5 min. at 4°C. The pellet was resuspended at a concentration of 1 mg wet weight/ml final volume in column buffer. Samples of 0.5 ml of this suspension were applied, chromatographed and collected as described for carboxylated latex microspheres above, except that fraction volumes were 100 drops (≈1.5 ml). Elution profiles were determined by measurement of absorbance at 300 nm as described for carboxylated latex microspheres.

Populations of VBS and VBI were prepared in column buffer as described in above, with egg yolk PC radiolabeled as described. Volumes of 0.5 ml of the uncentrifuged vesicles and Region III supernatents (see above) containing phospholipid concentrations of approximately 10 mM were applied to the S1000 column as described for *Seratia* above. Elution profiles were determined by scintillation counting of aliquots of all fractions.

High performance gel filtration chromatography was performed on an HPLC in the laboratory of Dr. Gregorio Weber and consisted of a Spectra Physics Organizer (8750-10, with Rheodyne 7125 injector and 50 ul sample loop), an Extended Range LC Pump and Solvent Delivery System (8700-XR) and a McPherson flow-through Spectrofluorimeter (F7749) fired by a 150 watt Xe short-arc Lamp Power Supply (750-03) and interfaced with a Spectra Physics recording Integrator (SP 4290). The 0.75 cm x 30 cm TSK-G6000 PW column (Toya-Soda, Tokyo, Japan) described by Ollivon *et al.* (134) was used.

A population of a VBS was prepared in 20 mM MES, 10 mM NaCl, 0.02% NaN_3 pH 6.0 according to the standard method, with egg yolk PC labeled with 0.5 mol% N-NBD-PE as described above. Uncentrifuged vesicles and Region III supernatents were diluted to concentrations of 1 mM. An aliquot of 50 µl was injected, and flow-through fluorometric detection (excitation 465 nm, emmision 535 nm) was initiated at a sensitivity of 0.03. Vesicles elution was with helium-purged 5 mM HEPES, 145 mM NaCl, 0.02% NaN_3 pH 7.4, at a flow rate of 1 ml/min. The duration of the analysis was approximately 15 min. No fractions were taken; analysis reported is based on the 2 ml/cm profile and other data recorded by the SP 4290 Integrator.

F. Assays of activity and protein concentration

Reported activities of PHLD are based on the two-phase assay of Clancy (31) as elaborated by Wissenberg (204). A 10 mM (1 x 10^9 dpm/mmol) preparation of DISP of [choline, N-C^3H_3] egg yolk PC in 25 mM NaCl were prepared for use as substrate (see Materials section above). Final assay concentrations were 500 µM PC, 200 mM acetate, 50 mM $CaCl_2$ pH 5.6. Enzyme was in-

cubated in the assay mix under 50 µl water- washed diethyl ether for 10 min at 30°C. The reaction was stopped by addition of 20 µl 5N HCl. Phospholipid was removed by extraction of the 0.22 aqueous phase (and 50 µl ether) with an additional 1 ml $CHCl_3$. Released [N-C^3H_3] choline was quantitated by scintillation counting of a measured aliquot of the aqueous phase. One unit of enzyme was defined as that quantity which catalyzed the hydrolysis of 1 µmol of PC to PA and choline in 1hr. at 30°C.

The qualitative colorimetric assay of Allgyer and Wells (5) was performed in order to determine activity-containing fractions in column fractionation steps during protein purification. An assay mix composed of 1 mM dihexanoyl PC, 50 mM $CaCl_2$, 0.5 mM dithiothreitol, 0.1 mM methyl red pH 5.6 was dispensed into the 0.1 ml wells of a microtiter plate. Aliquots of 10 µl from individual column fractions were added to each 0.1 ml well, andthe reaction was allowed to proceed for 15 min. at room temperature. Fractions containing phospholipase D activity developed a red color during this time period. Activity-containing fractions were then reassayed according to the quantitative assay described above.

Protein concentrations were determined by the method of Lowry *et al.* (118) using bovine serum albumin as standard. Precipitation of protein with 6% trichloroacetic acid was used when the samples contained interfering compounds. Protein concentration profiles for some column chromatography experiments were produced using the Coomassie Brilliant Blue staining procedure of Bradford (20). A stock solution of the Coomassie reagent was prepared in a volumetric flask by dissolving 100 mg in 50 ml methanol, adding 100 ml 85% phosphoric acid and diluting to 200 ml with water. In a typical experiment, 100 µl was transferred from each even-numbered column frac-

tion to a disposable 1-ml cuvette (Sarstadt). A 1 ml volume of a 1:5 dilution of the stock Coomassie reagent was added and the samples were mixed and incubated at room temperature for 10 min. Optical density at 595 nm was measured to provide relative protein concentrations.

G. Purification of Phospholipase D

Three preparations of Savoy cabbage Phospholipase D (PHLD) were used throughout the course of this work. Reference to commercial PHLD is to product P-7758 from Sigma Chemical (0.45 units [μmol min^{-1}] and 0.55 mg protein per mg material), which was most often used without further purification. Acetone precipitated PHLD refers to enzyme purified through the acetone precipitation step of Yang (207, described by Clancy, ref. 31). The activity and proportion of protein in this preparation was 0.15 units and 0.40 mg protein per mg material, respectively. Affinity-purified PHLD refers to a preparation purified from the acetone precipitate by ammonium sulfate precipitation (31) and by affinity chromatography (Allgyer and Wells, ref. 5). Affinity purified-enzyme typically were found to have 18 - 30 units and ~6 mg protein per ml (specific activity, 3 - 5 units/mg). The commercial and acetone-precipitated forms were stored as lyophilized powders, or, as the affinity-purified PHLD, at 6 - 15 mg/ml concentrations in 80 mM TES, 2 mM EDTA, 0.04% NaN_3 pH 7.25/ethylene glycol (1:1).

Ethylene glycol (EG) was removed on a weekly basis by gel filtration chromatography on 20 ml Sephadex G25 in 1.5 cm x 12 cm column as described in the Section on VBI above; elution was with 20 mM TES, 10 mM EDTA 0.3 M *myo*-inositol pH 7.25 (TES-Inositol). In cases where protein concentration was critical, ethylene glycol could be removed by vacuum dialysis (described above) against TES-Inositol at 4°C.

Some variations in the above methods were required with changes in the scale of purification. A commercial scale food processor in the Food Science Laboratory, Department of Foods and Nutrition was used in preparations starting with 20 Kg or more of starting material. The Sharples continuous flow centrifuges of the Department, and of the Department of Microbiology were used for initial centrifugation steps. Moreover, several refinements of the methods were investigated. Initial homogenization was performed in 40 mM TES, 0.3 M inositol pH 7.5. Resuspension of the acetone-precipitated pellet prior to lyophilization was done by stirring with several smaller volumes, for shorter time periods. In instances when acetone precipitated PHLD solution contained significant quantities of green pigment, the lyophilized material was extracted with one volume of water-washed diethyl ether (5) at 4°C. In such cases, it was necessary for the powder to be extremely dry; it was therefore lyophilized for an extended period at room temperature.

In some cases the ammonium sulfate precipitated enzyme solution was applied to columns of Biogel AcA 34, Sephadex-200 or Biogel P-150 for fractionation according to size (see refs. 31 and 5). Typically, 85 ml gel was swollen, packed and equilibrated with TES-EG buffer in a 1.9 cm x 35 cm. Samples were applied without flow-adaptors, as described in the Section on Sephacryl S1000 above, and were eluted with TES-EG at a flow rate of 0.6 ml/hr. For the AcA 34 and G-200 columns, and 0.14 ml/hr for the P-150 column. Protein was followed by flow through absorbance at 280 nm on the Altex instrument of Dr. John Clark described above, and enzymatic activity by the qualitative assay described below. Active fractions were pooled and concentrated to approximately 2 ml by ultrafiltration on an Amicon PM-30 membrane, or by vacuum dialysis in the Prodicon unit described above.

Preliminary batch experiments were performed using a phenyl-Sepharose hydrophobic interaction chromatographic material from Pharmacia. Experiments were performed according to manufacturer's recommendations on PHLD purified through the ammonium ammonium sulfate

precipitation step. Residual ammonium sulfate from the salting-out process was used to drive the hydrophobic association.

Commercially available γ-aminopropane agarose from Sigma Chemical with 5 μmol reactive amine per ml settled gel was eventually substituted for gels synthesized by the procedure of by Dr. Robert Clancy (original activity, 0.3 μmol/ml settled gel). Column fractions containing affinity purified enzyme were pooled; concentration of enzyme and removal of EG were accomplished simultaneously by vacuum dialysis (as described above for vesicles) against 5 mM TES 0.3 M inositol pH 7.5 in the ProDiCon.

H. Other Enzyme Characterization

Sodium dodecyl sulfate-poly(acrylamide) gel electrophoresis was performed using slab gels of 7.5 and 15% (w/v) acrylamide according to the method of Laemmli (106). Cylindrical non-denaturing poly(acrylamide) gels electrophoresis was performed according to the standard laboratory method (provided by R. Huss). Slab gels were stained for 3 hours in 0.1% Coomassie Brilliant Blue, 25% isopropanol, and 10% acetic acid and destained in 20% isopropanol and 8% acetic acid, both in distilled water. Gel shrinking was performed in an aqueous solution of 65% methanol, 0.5% glycerol overnight. Gels were then air-dried between cellophane sheets on a home-built plexiglass rack. Non-denaturing gels were sliced (Bio-Rad), and activity was eluted in TES-EG.

Stock solutions of PHLD were periodically analyzed for extractable lipid and pigment. Typically, 50 μl of PHLD stock solutions was combined with 650 μl distilled water and 100 μl 6N HCl. This volume was then extracted according to the procedure of Bligh and Dyer (19) described

below. The organic phase was dried under nitrogen, redissolved in minimal chloroform, and chromatographed and on a one- or two-dimensional phospholipid plate as described below.

I. Extraction and quantitation of PHLD reaction products

Phospholipids were extracted from post-reaction mixtures as described in the Clancy assay (see the PHLD Section above, and ref. 31) or by the method of Bligh and Dyer (19). In both cases, 0.1 volume 5N HCl per volume aqueous mixture was added to stop the reaction. In the former case, volumes of aqueous, diethyl ether and chloroform were held in constant proportion at 22:5:100, respectively, and the extractions were scaled as needed. In the latter case, samples from 0.2-2.0ml were extracted. $CHCl_3/CH_3OH$ (2.5 volumes, 1:1) was added and the sample was vortexed for 30 sec. Vortexing was repeated after subsequent additions of 1.25 volumes each of $CHCl_3$ and water. Phases were separated by low speed centrifugation in a bench top clinical centrifuge. When aliquots were desired from the aqueous phase of either assay, sampling was done immediately. When aliquots were desired from the lower organic phase, sampling was done by Hamilton syringe after removal of the aqueous phase and after a 1 ml water wash.

Analyses of phospholipids by two-dimensional thin layer chromatography were performed according to the method of Rouser (156). Plates of silica Gel G (250 μm, 10 cm x 20 cm from Analtech, Newark, DE) were pre-run in acetone, and air dried; samples were applied by 50 μl Hamilton syringe. Loading for purity analyses were typically 200 μg. The first dimension was developed with chloroform/methanol/water (65:25:4) and the second by n-butanol/glacial acetic acid/water (3:1:1), with drying of the plate between dimensions. Single dimension separations were performed using the first dimension of this system. Phospholipids were visualized with iodine vapor, with the reagent of Dittmer and Lester (48) or by warming after H_2SO_4 spray.

Quantitation of phospholipid or phospholipid phosphate in iodine-visualized spots of TLC was performed after elution of compounds from silica. Silica with bound phospholipid was scraped from plates into a 15 cm glass elution column plugged with glass wool, and phospholipids were eluted with 5 ml of chloroform/methanol/glacial acetic acid/water (5:5:1:1, v/v) followed by 2 ml methanol. Solvent was removed under nitrogen and samples were resuspended in 0.2-2 ml 0.001 N HCl with rotary mixing. Phospholipid was then extracted by one of the methods described above, and taken to dryness under nitrogen. Alternatively, phospholipid extraction from silica based on the method of Kolarvic and Fournier (100) was tested. Silica was scraped into 22 mm glass scintillation vials, followed by the addition of 400 µl of water and 10 ml of hexane/isopropanol (3:2, v/v). After agitation, the silica was allowed to settle, and a measured volume of the liquid as transferred to a second glass scintillation vial and taken to dryness.

Phospholipid concentrations were determined as inorganic phosphate after perchloric acid ashing according to the method of Kates (93), as intact phospholipid according to the method of Stewart (177), or by scintillation counting as described above. Phospholipid peroxidation was monitored by measurement of optical density at 233 and 215 nm as suggested by Klein (99) and Shaw and Thompson (169). Phospholipids with Klein index values (OD_{233}/OD_{215}) of less than 0.2 were accepted as relatively free of peroxidation products.

J. High Resolution Phosphorus-31 and Carbon-13 NMR Spectroscopy

1. Phosphorus-31

Most ^{31}P NMR experiments were performed on the NSF 250, a multinuclear wide-bore super-conducting Fourier-transform (FT) NMR instrument, a part of the National Science Foundation (NSF) Midwest Regional Instrumentation Facility (RIF). System components are

from Oxford Instruments (magnet, probe and shim supply), Nicolet (pulse programmer, data processor, software and plotter), Cryomagnet Systems (field/frequency lock) and Varian (variable temperature controller). An HP5105A frequency synthesizer and an Amplifier Research 10L amplifier were used in the proton decouple channel: a PTS 160 frequency synthesizer, an ENI 3100L rf power amplifier and an E0 250 multinuclear receiver were used in the observe channel. Data acquisition and processing was controlled by Nicolet 1180 software. Phosphorus observe and proton decouple frequencies were 101.265 and 250.158 MHz, respectively.

Experiments were standard single pulse as performed by the 1PULS program of the Nicolet 1180 Software. Two sets of pulse parameters were typically employed. Unless otherwise noted, references to 20 μsec pulse-width (PW) experiments refer to instrumental conditions of a 10 KHz sweep-width, with 8K data points in the frequency domain (DP), a 50 μsec pre-acquisition delay (DE), an approximate acquisition time (AT) of 0.41 msec and a 1.5 sec post-acquisition delay (D5). Recycle time for 20 μsec pulse-width experiments was 0.75 sec. Unless otherwise noted, references to 25 μsec pulse-width experiments refer to instrumental conditions of a 5 KHz SW with 8K DP, a 40 μsec DE, an approximate AT of 0.82 msec and a 1.0 sec D5. Recycle time for 25 μsec pulse-width experiments was 2.00 sec. Decoupler power for both 20 and 25 μsec experiments was ≈ 0.8 watts (unless otherwise noted); gated decoupling was accomplished using a Waltz 16 supercycle decoupler sequence and software from the standard Nicolet package.

The following is an outline of the elements of the instrumental method for ^{31}P experiments on the NSF 250. After the sample was introduced, the instrument circuitry (preamps, capacitors and filters) and observe channel components were set to observe the deuterium signal. The channel was then tuned, using the deuterium signal. Experimental conditions suitable for the observation of deuterium were then entered from keyboard, audio filters were then set based on desired sweep

width and other instrument parameters (46, 60), the observe amplifier was turned on, and signal intensity was adjusted (using gain and attenuation controls) using a repetitive single aquisition "link". Field homogeneity (the setting of each spinning shim) was adjusted based on visual inspection of the free induction decay (FID).

A short data acquisition was then used to generate a deuterium spectrum (see below for data processing). The ^{2}H line shape and width are noted (as an indication of the homogeneity of the magnetic field), and the shim routine was repeated if necessary. Typical deuterium linewidths after shimming were 0.5 - 1.5 Hz.

The observe amplifier was then turned off, and instrument circuitry and observe channel components were set (as described above) to observe phosphorus. The decoupler channel of the probe was tuned by minimizing reflected power, and decoupler power was set and Waltz 16 decoupling was selected. Experimental conditions for the phosphorus experiment (described in detail above) were then entered from keyboard.

Audio filters were then set, the observe amplifier turned on, and signal intensity was adjusted (as for deuterium above) using the repetitve single aquisition "link". Data was then acquired using a link and the 1PULS program. After acquisition was completed, signals were conditioned by a line-broadening routine involving exponential multiplication of the FID. A Fourier transform of the data was then performed, the phase of the frequency spectrum was adjusted, and the spectrum was plotted, noting linewidths, integrals, etc.

The same outline was used for a single experiment, performed on a Varian XL100, a multinuclear wide-bore FT instrument with fixed electromagnet, previously a part of the NSF Midwest RIF.

The XL100 ^{31}P probe used an internal deuterium lock. Phosphorus observe and proton decouple frequencies were 40.503 and 101.258, respectively. Full and gated proton decoupling were used.

2. Carbon-13

All ^{13}C experiments were performed on the NT 360, a Nicolet pulsed narrow-bore super-conducting FT NMR instrument equipped with NIC 293B pulse programmer, NIC 1280 data system and CDC Hawk disk drive, also apart of the NSF Midwest RIF. The probe used had an internal deuterium lock. Data acquisition and processing were as above. Full and gated proton decoupling experiments were performed at ambient temperature.

3. Miscellaneous

Sample spin rate was approximately 20 cps. The number of aquisitions and line-broadening affected by signal conditioning are as noted in the figure legends. Chemical shift referencing was to external phosphoric acid, 85% in $^{2}H_2O$; the instrument was retuned and reshimmed after change of sample. ^{13}C chemical shift referencing was to published values of the carbon resonances of the buffer used for the experiment (TES; see ref. 18).

K. NMR Sample Preparation

All ^{31}P sample volumes were greater than 3.5 ml as required by the geometry of the probe used. Samples of less than 5 ml were run with a finned poly(tetrafluoroethylene) vortex plug (Wilmad) to prevent wall-climbing by the spinning sample. Phosphorous acid, trimethyl ester was dissolved in CD_3OD to a final concentration of 1% (v/v). The glycerophosphate (GP) sample was 25 mM GP

and 20 mM EGTA in D_2O, with pH adjusted to 5.6 with deuterated acid and base. Measurement of pH here and below was done on a standard pH meter without correction for deuterium effects. Phosphorylethanolamine was prepared at a concentration of 60 mM in 20% ^2H_2O in glass-distilled water, and the pH of portions of the stock were adjusted to 3.1, 5.6 and 7.9.

Homogeneous VBS were prepared according to the standard method described above. VBI were prepared according to the standard method except that injection volumes varied from 0.1 to 4 ml as noted, and the final concentration of ethanol in encapsulation buffer was 25% (v/v). VBI were chromatographed on Sephadex G25 to remove ethanol and fractions absorbing at 300 nm were pooled and concentrated to approximately 30 mM by vacuum dialysis against isotonic buffer. VBS and concentrated VBI were centrifuged to obtain homogenous populations of vesicles. See preceding Methods sections above for further preparative details.

As noted in the figure legends, standard final buffer compositions for both VBS and VBI were generally 20 mM PIPES, 13.5 mM NaCl, 5% ^2H_2O (v/v), pH 6.7 or 10 mM TES, 13.5 mM NaCl, 1% ^2H_2O (v/v) pH 6.5. Buffers are noted by the cation and the buffering agent present, e.g., Na-TES, Pr-PIPES, etc.

L. Evaluation of the Nuclear Overhauser Effect in Phosphorus-31 NMR Spectra.

The Nuclear Overhauser Effect (NOE) was predominantly evaluated using the gated decoupler using the Waltz sequence. The NOE was measured by comparison of changes in intensity found when comparing fully decoupled spectra to those acquired with gating of the decoupler pulse (in order to attenuate the NOE, ref. 58). Probe temperature was ambient for all experiments. Intensities were evaluated by comparing one or more of the following (listed in order of increasing

reliability): (i) peak heights, for resonances with line-widths less than 1 Hz, (ii) areas calculated by triangulation, (iii) integral values calculated by the Nicolet 1180 software, (iv) weights of resonances cut from photocopies of spectra.

M. Resolution of Inner and Outer Leaflet Resonances

Unless otherwise noted, all vesicle experiments were performed with gated decoupling as decribed for NOE measurement above. Probe temperature was held constant at a temperature above 20°C for VBS and VBI of egg yolk PC, at $26^0 \pm 1$°C for DMPC VBS and at 33 ± 1°C for DMPC/DMPA (9:1) VBS. Addition of shift reagent was accomplished by (i) preparation of vesicles in the presence of the reagent, (ii) preparation of the vesicles in the abscence of reagent, measurement, addition of a concentrated reagent, retuning and reshimming, measurement, etc. or by (iii) preparation of the vesicles in the presence or abscence of reagent, followed by vacuum dialysis to replace the external cation. Care was taken to match ionic strength of aqueous compartments (32).

N. Spectrophotometry and Spectrofluorometry

Absorbance spectra of carboxylated latex microspheres diluted in SDS-column buffer (see below) over a range of 1:10 to 1: 50,000 were measured from 190 nm to 800 nm in 1 cm quartz cuvettes in a Hewlett Packard 8451A Diode Array Spectrophotometer with slits set at 2 nm in the laboratory of Dr. Lowell Hager. Fluorescence emission spectra of similarly prepared samples of coumarin-labeled carboxylated latex microspheres were measured from 350 nm to 650 nm in 1 cm quartz cuvettes in an SLM 8000 Scanning Spectrofluorometer controlled by an Apple IIc computer in the laboratory of Dr. Enrico Gratton, Department of Physics.

Measurement of steady-state fluorescence depolartization was performed according to methods standard in this laboratory, based on work from the laboratory of M. Shinitzky (170). This work was performed on an SLM 4000 Spectrofluorometer (SLM Instruments, Urbana, IL) in the ratiometric and integrating mode. Excitation for both DPH and TMA-DPH was 360 nm. A Schott 7-54 filter was used in the excitation path, and a 2M $NaNO_3$ filter was used in the emission path to remove wavelengths below 390 nm (171). The depolarization was calculated using the equation :

$$P = \frac{(I_{par} / I_{perp}) - 1}{(I_{par} / I_{perp}) + 1}$$

where I_{par} is the fluorescence intensity parallel to and I_{perp} is the fluorescence intensity perpendicular to the plane of polarization of the excitation beam (170). Anisotropy values, r, are calculated from the polarization values using the equation: $r = 2P/(3 - P)$. Each polarization value was calculated from the mean of three acquisitions, each the integral of a sampling of greater that 50 data points. See Lakowicz (108) for a thorough discussion of the method and calculations.

For the experiments reported here, DPH and TMA-DPH were added exogenously, from 20 mM stocks in tetrahydrofuran. Solution concentrations of both probes were determined using the extinction coefficients for each at 350 nm and 25°C in dimethylformamide (Prendergast et al., ref. 141): $\varepsilon_{DPH} = 81{,}000$ liter mol^{-1} cm^{-1} and $\varepsilon_{TMA\text{-}DPH} = 30{,}200$ liter mol^{-1} cm^{-1}. Small aliquots were added to vesicle solutions such that probe to phospholipid ratios were 0.001 and 0.002 for DPH and TMA-DPH, respectively. See figure legends for buffer compositions. Temperatures were varied by circulation (at the adjusted temperatures indicated in the figures) using a Lauda K-2/r circulating water bath. Temperature was stable over the course of each set of measurements. Samples were incubated at 37°C for 15-45 min. at the beginning of each

experiment to insure uptake of the probe. In all cases, photon counts were stable at the onset of each measurement at each temperature.

Data for producing laurodan fluorescence spectra were acquired and processed on either the SLM 8000/Apple 2c described above, or on a Spex system equipped with an HIPLOT Digital Recorder, in the Department of Chemistry. In both cases, excitation wavelength was 350 nm, and emission was scanned from 360-650 nm. Slit width on the Spex instrument was 0.5 mm, and acquisition and integration times were 0.5 sec. In the latter case, data were smoothed by an averaging process using the algorithm of Savitsky and Golay (157). The number of points averaged are indicated by the smoothing factor (SF) indicated in the figure legend.

O. Bipolar Pulse Conductance of Phospholipase Reaction Products

Computer-controlled bipolar pulse conductance measurements were performed in collaboration with Drs. Douglas Taylor and Timothy Nieman, Department of Chemistry, to assess the possible application of the phospholipase C and phospholipase D (PHLD) reactions as immobilized enzyme detectors for flow-through conductiometric detection of phospholipids in HPLC (184). The buffer for phospholipase C related measurements was 50 mM Tris, 6.3 mM $CaCl_2$, pH 7.3 at 37°C, while for PHLD it was 200 mM acetate, 50 mM $CaCl_2$, pH 5.7 at 25°C (71). Phospholipase C related samples contained 2.5 mM DMPC and 2.5 mM each of 1,2-dimyristoylglycerol and phosphorylcholine, corresponding to pre- and post-reaction conditions, respectively. The PHLD related samples contained 0.5 mM egg yolk PC and 0.5 mM each of egg yolk PA and choline chloride, again corresponding to pre- and post-reaction conditions, respectively. All samples were dispersed by sonication (as described for VBS above) for 5 min. at their assay temperatures. Theory and instrumentation for bipolar pulse conductance measurements has been described (184).

P. Data Analysis

Absorbance and radioactivity data for samples eluted from Sephacryl S1000 columns were normalized by dividing measured radioactivity of each fraction by the radioactivity of the peak fraction. Total radioactivities of chromatographed samples so compared were generally within 30%. When a sample profile clearly indicated a peak maximum at elution volume offset from the maximum measured radioactivity, the elution volume corresponding to the true maximum was estimated by the graphical method of tangents. All such estimations are discussed in Results and Discussion. For a description of the data analysis involved in radioactivity counting of samples containing both ^{3}H and ^{14}C see Methods.

Simple mathematical functions as well as linear regression, standard deviation and analysis of propagated error were performed on a Hewlett Packard 10C calculator. Discussion of fluorescence polarization data is based in part on results of simple and exponential fitting by routines of Cricket Graph Release 1.2. Data plotting software was the Cricket Graph routine mentioned (CG), was from Mr. David Chambers and Dr. James Phillips of the Department of Theoretical and Applied Mechanics, University of Illinois (TAM) or was the "lstsqb" program in Supercalc3 (version 2.1) from Computer Associates (San Jose, CA). A Macintosh II microcomputer with hard disk and Imagewriter II printer was used to run the CG program, and an IBM PC-AT microcomputer with hard disk was used to process data and run the TAM and SC3 programs, where data was plotted by Hewelett-Packard 7475A and 7470A recorders, respectively.

Elution volumes from chromatographic analysis by HPLC on the TSK G6000 PW column are as reported by the Spectra Physics SP 4290 integrator. Yield for that analysis is calculated from integration values of the two traces, again as reported by the SP 4290. The values of $w_{1/2}$ for the analysis were measured from SP 4290 traces.

IV. Results and Discussion

A. Methods to Produce Large Diameter Vesicles

1. Phospholipids

Several methods were investigated and developed to produce unilamellar vesicles for later physical study. For each method, commercially available or easily isolated phospholipid was used, typically DMPC or egg yolk PC. Early experiments suggested the importance of noting phospholipid purity prior to and following vesicle preparations. TLC analysis of certain freshly opened commercial preparations of "egg yolk PC" indicated the presence of at least two phospholipids by I_2-staining, as well as significant impurity migrating as neutral lipid at the solvent front (data not shown).

More clear and ubiquitous was a fluorescent contaminent of a variety of commercial PE preparations, observed by Lane Conn in this laboratory to comigrate with PE in several TLC solvent systems. The contaminent was also present in bacterial and mammalian phospholipid isolates, and evidence eventually lead Conn, Geneva Omann and others to the conclude that it was a product of auto-oxidation of the polyunsaturated fatty acids of natural PE.

Egg yolk PA was found to be particularly susceptible to oxidation during normal handling, especially as its free acid form. Use of the free acid was required because the sodium salt of PA was not soluble in the nonpolar solvents specified in some methods. Mixing in a small percentage of a polar co-solvent aided in dissolving the PA, but also changed significantly the conditions in which vesicles were formed. Consequently, regular use of a polar co-solvent would have required the rigorous characterization of vesicles required for any new method. Instead, extraordinary care was taken with the PA sodium salt preparations, and otherwise pure commercial and extracted preparations were discarded if solutions were judged to be significantly oxidized.

The instability of fatty acids of naturally occuring phospholipids was of concern in light of the observed effect of peroxidation on both the biological and physical properties, and on the transbilayer distribution of phospholipids in vesicles. Klein (99) noted that "if air is present during the preparation of liposomes from phospholipids of biological origin by sonication in an aqueous system, the K^+ permeabilities measured for different preparations are not reproducible," and reported that reproducibility improves if sonification is under nitrogen. Shaw and Thompson (169) observed that an otherwise stable asymmetry in VBS of [N-methyl-C^2H_3] PC decays rapidly when the concentration of phospholipid oxidation products reaches a critical level. That is to say, the accumulation of oxidative impurities results in a condition where flip-flop of PC can occur. Other reports of the effects of lipid peroxidation and of the implications of this phenomenon have been discussed and reviewed by Schroeder (162, 152).

In light of the possible influence of peroxidation, and having observed oxidative changes in VBS composed of egg yolk PC/PA in the course of two day experiments, the added precautions described above were used in handling phospholipid and vesicle preparations. Furthermore, as is

noted below, consideration was given to more rapid methods of vesicle preparation, allowing physical measurements to be performed on the day of preparation.

Apart from these considerations, phospholipid techniques were nearly always those standard to the laboratory. Alternative methods of extraction (100), phospholipid quantitation (27, 177) and visualization after TLC analysis (48) were evaluated, and at times were found to be useful. Such exceptions are noted in the descriptions of the experiments in Methods and in the figure legends.

2. Bipolar Pulse Conductance

Thought was given to the use of HPLC in phospholipid purification and analysis. HPLC separation methods are available for phospholipid mixtures, but their use is qualified by the problem of detection of low levels of separated species. Conversations with Dr. Douglas Taylor led to preliminary experiments investigating the possible use of computer-controlled bipolar pulse conductance for the indirect detection of eluted phospholipids. Taylor and Nieman (184) have shown that amino acids can be detected by comparative measurement of the bipolar pulse conductance of solutions prior to and following oxidation catalyzed by amino acid oxidase , based on the changes in conductivity resulting from production of corresponding 2-oxo acids and ammonia. Their observations were practically applied to the detection of amino acids by placement of a flow-through model of this type of conductance detector, a short column of immobilized oxidase (covalently linked to controlled-pore glass), and a second conductance detector in series after the analytical column in an HPLC system.

The products of the phospholipase C and phospholipase D (PHLD) catalyzed reactions include a phosphoryl species which may be expected to alter the conductivity of its solution with respect to pre-reaction conditions. Solutions reflecting pre-and post reaction conditions were prepared based

on the standard methods for assaying these two enzymes (71). Measurements of the bipolar pulse conductance were hampered, however, by the instability of suspensions of the amphipathic reactants and products in the preparations. The experiments were discontinued in light of the need to identify solution conditions which simultaneously satisfy the solution requirements for phospholipid separation, enzyme activity and conductivity measurement (184). While this specific coupling of immobilized-enzyme (phospholipase C or PHLD) treatment and electrochemical measurement may not prove applicable to phospholipid detection, the use of a stable, immobilized lipolytic enzyme, or any other post-separation modification scheme, coupled with electrochemical measurement, may be worth considering in answer to the question of phospholipid detection.[1]

3. Vesicle Preparations

Specific methods considered for vesicle production are listed in Table 6. Of those listed, dispersions (DISP), vesicles by sonication (VBS) and vesicles by injection (VBI) were routinely used to prepare vesicles for experiments. Vesicles by detergent dialysis were routinely prepared by Mr. Joseph Cimino for use in studies of protein-lipid interactions. Other methods were used only initially to assess their convenience as regular preparative methods. Comments contained in the Table are based both on these preparative experiences and on published reviews (181, 42) and reports (noted in Methods and below). The purpose of their assessment was to arrive at a method which could be used to produce greater than 80 nm diameter unilamellar vesicles on a daily basis for use in subsequent studies of the physical properties of asymmetric membranes. Final concentrations of approximately 30 mM and the capability of producing vesicles of varying diameters were other features desired. While criteria such as trapped volume and encapsulation

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[1]A recent report accomplishes this using phospholipase D: see Chemical Abstracts, CA10611083095.

Table 6 Comments on Methods of Vesicle Preparation Reported in Materials and Methods.[a]

Vesicles by:	Means of Dispersing Phospholipid	Comments[b]
dispersion, DISP	ultrasound	*multilamellar;* duration of hydration manner of dispersing critical
sonication, VBS	ultrasound	relatively homogenous; *minimal diameter;* duration and manner of sonication critical; physical properties, transbilayer phospholipid symmetry by affected by curvature
fusion	ultrasound	weeks to prepare; limited to saturated (synthetic) phospholipids
evaporation	ethers	concentrates solubilizer impurities; vacuum control and sample attention critical; possible introduction of oxidizing via solvent
effusion	alkanes	*maximum concentration ≤15 mM;* may concentrate solubilizer impurities; requires injector; uncharged vesicles may aggregate
detergent dialysis[c]	detergents	days to prepare; concentrates solubilizer impurities; rate of dialysis critical; requires large buffer volumes; suited for membrane protein reconstitution
injection, VBI	ethanol	*maximum concentration ≤7.5 mM;* may concentrate solubilizer impurities

[a]Unless otherwise noted, all methods can be used to produce unilamellar vesicles with diameters of ~80 nm, at phospholipid concentrations of ~30 mM.

[b]Based on preparative experience, and published reviews and reports.

[c]Routinely prepared by Mr. Joseph Cimino.

efficiency are of primary interest when evaluating vesicles for use as vehicles for drug delivery (181), they are of limited interest here and are not included in the Table.

a. Vesicles by Dispersion and Sonication

Dispersions (DISP) are formed spontaneously when dry phospholipid is agitated in the presence of an aqueous phase. Vesicles by sonication (VBS) are formed when DISP are exposed to ultrasound for a somewhat extended period at a temperature above the T_c of the highest melting component. Both DISP and VBS were routinely prepared and are included in the Table because of their later use as controls. The adaptations to the published methods for producing these two types of vesicles described in Methods were chosen as an added precaution against phospholipid contamination and degradation, or simply for the sake of convenience.

For DISP, duration of hydration and manner of dispersing were held constant from experiment to experiment because of the known impact of these two parameters on vesicle structure (81). Brief sonication of DISP decreases the mean diameter and narrows the size distribution in proportion to energy and duration of sonication, and unless carefully controlled is a source of variability among preparations (11). Consequently, this method to aid in dispersing phospholipid was not used. Producing DISP as described has been reported to result in a population with mean diameter of 400 nm, with an average of approximately 10 lamellae per vesicle (81).

Descriptions of the physical properties of DISP and VBS, are referred to in the Introduction. As noted, the physical properties are dependent upon diameter and on the number of lamellae, in addition to recognized parameters of composition and temperature. As noted, DISP are heterogenous and multilamellar, with diameters generally much greater than 80 nm. VBS are rela-

tively homogenous, highly-curved unilamellar vesicles; mean diameter is noted consistently in the literature to be on the order of 22 nm (82, 151). The multilamellar nature of DISP and the implications of high curvature of VBS make these vesicle types less useful for studies of phospholipid dynamics in asymmetric membranes.

b. Vesicles by Fusion

Vesicles by fusion are formed when the thermodynamically less stable bilayers of two or more VBS fuse to form a single, large-diameter structure. Generally, the term will be used to refer to the two populations of vesicles available after incubation of high concentrations (≈100 mM) of VBS of DMPC and dipalmitoyl PC at slightly acidic pH at 4°C. Under these conditions, VBS fuse to form a population of 70 nm vesicles, corresponding to nearly 100% of the initial phospholipid after 7 days. Incubation through 35 days results in a population of 95 nm vesicles, again corresponding to nearly 100% of the initial phospholipid (see Wong *et al.*, ref. 206).

One requirement for vesicle fusion appears to be near neutral or slightly acidic conditions. A buffer pH of 6.0 was selected for that reason, and because pH 6.0 is the intersection of pH PHLD stability and activity curves (31). It is therefore a reasonable pH to consider for longer enzyme-vesicle incubations. While a preparation of vesicles by fusion takes weeks to prepare, the amount of committed time required is very little and the method is quite simple. In addition, the method does not involve solvent or detergent removal, and is therefore free of impurities which might be concentrated from these sources (138). Unfortunately, this method is apparently limited to phospholipids with a T_c of less than 4°C, i.e., PC with saturated fatty-acyl chains.

c. Vesicles by Evaporation

Vesicles by evaporation are reportedly large unilamellar and oligolamellar vesicles which form upon reorientation of dissolved phospholipid in an organic-in-aqueous emulsion upon the evaporation of the organic phase. Application of reported methods for producing vesicles by evaporation, the "reverse-phase" vesicles of Szoka and Popahadjopoulos (180), to saturated phospholipids, specifically DMPC, requires both lower concentrations than the general method, and the use of a chloroform-containing solvent (179). Several trial experiments were performed with DMPC in diethyl ether and diisopropyl ether, alone and in combination with chloroform and methanol, to determine solubilities. DMPC did not completely dissolve at or above 20 mM in diethyl ether, the general conditions suited for mixtures of egg phospholipids (180). Furthermore, DMPC failed to completely dissolve at concentrations of 12 mM or greater in ≈60% (v/v) chloroform in diethyl ether. Concentrations of 20 mM in ≈20% (v/v) methanol in diethyl ether were found to be suitable in terms of DMPC solubility, but questionable in terms of the added miscibility with water introduced by the use of methanol. DMPC was found to be soluble at a concentration of 10 mM in diisopropyl ether-chloroform, (1:1) conditions which may be optimum for saturated phospholipids.

As has reported, the ratio of vessel surface area to total volume was seen to be critical in determining the rate of solvent removal during evaporation (179). Screw-cap tubes were found to be difficult to work with because vessel geometry made slow, careful removal of the volatile diisopropyl ether-chloroform solvent almost impossible, and because thick walls hindered effective cup-horn sonication. Use of the various commercially available small round-bottom flasks circumvented the former but not the latter difficulty. Consequently, round bottom flasks with ≈5 ml volumes with very thin walls (<1 mm) were custom-blown by Mr. Donald O'Brien and colleagues in the glass shop in the Department of Chemistry; screw cap closures were chosen so

that the preparation vessel could be used for routine use and storage. The actual diameter of 5 ml round-bottom flasks was chosen to give a surface area-to-volume ratio of ≈40 cm^2/ml. Egg yolk PC experiments in diethyl ether were performed in similar vessels with a surface area-to-volume ratio of ≈22 cm^2/ml, reflecting the smaller volume of organic solvent required for egg yolk phospholipids.

Following formation of the aqueous-in-organic emulsion by brief sonication, evaporation was performed in two steps at somewhat discrete levels of vacuum (400 and 730 mm Hg, ref. 179). Attention during the evaporation stages is critical; the change to lower vacuum (higher pressure) must be made when the gel stage is reached. Bumping, and, in general, rapid solvent removal must be avoided because of sample loss and DISP formation (179). Water aspiration and house vacuum were found to be ill-suited as vacuum sources because of extreme fluctuations in vacuum level. A vacuum pump was used as a constant source of high vacuum, and the level of vacuum was adjusted somewhat successfully by metering a flow of nitrogen into the rotary evaporator. The use of a McLeod manometer and Lewis manostat (202) or other comparable measurement and metering devices were considered because of the critical nature of the evaporation step, and the detrimental effects of gas pumping on the vacuum pump.

Initial solvent purity and solvent removal are critical for producing vesicles by evaporation free of oxidation products and other contaminents. The peroxides that are slight degradative contaminents in commercial ethers accumulate with time; the use of anhydrous ethers (lacking ethanol or other preservatives) exacerbate the problem. It is at least necessary to extract ethers with water prior to use; most reports suggest distillation from sodium bisulfite before use.

The lack of such precautions has been shown by Parente and Lentz (138) to influence the physical properties of the vesicles produced. These authors note that even carefully prepared vesicles by

evaporation lack the highly cooperative phase transition generally observed for pure phospholipid vesicles by differential scanning calorimetry. From the increased breadth of the main transition of vesicles of a number of natural and synthetic phospholipids, these authors estimate the presence of ≈30 mol% of contaminant. They suggest that in this and similar methods, the phospholipid tends to solvent-extract and concentrate any lipid soluble impurities present in the evaporating organic solvent. Furthermore, residual solvent has been shown to remain dissolved in the hydrophobic region of the membrane. Removal by chromatography or dialysis is necessary, with the disadvantages of increased time and sample dilution and loss.

Another question commonly raised with methods involving sonication and organic solvent exposure is their applicability to membrane protein reconstitution, especially in light of the protein denaturing effect of many organic solvents. While application to protein reconstitution was not an immediate concern, its potential would be an added benefit. In this respect, vesicles by evaporation have apparently been used successfully to reconstitute bacterial rhodopsin without significant change in spectral characteristics, and its use with methods involving Triton X-100 solubilized proteins has been suggested (181).

d. Vesicles by Effusion

Vesicles by effusion form when phospholipids in a low-boiling organic solvent reorganize as the solvent is effused through an orifice into an aqueous medium, and evaporates because of increased temperature or decreased pressure. Here, the slow infusion of PC was accomplished manually, although the automatic injector described below for vesicles by injection (VBI), below, is better suited for reproducible injections. Temperatures required for evaporation of solvent are generally in excess of T_c+10°C, and when a Liebig condenser serves as injection vessel (41) this temperature is easily maintained by water circulation as described for VBS production. Addition of a cold

condenser surmounting the first reduces loss of aqueous volume by evaporation (50). This loss is problematic since the aqueous volume is purged by a continuous stream of inert gas to prevent peroxidation, and to aid in mixing and reduce aggregation. The added extrusion used in this method is not the later-developed technique for producing homogenous populations based on size; rather, it serves to remove extremely large aggregates of phospholipid that may form as a consequence of the infusion.

The use of high temperature and its consequences for protein denaturation and solute decomposition can be avoided by infusion at 30°C under reduced pressure (41). Vacuum infusion required very small orifices, here custom drawn from glass, to prevent too rapid addition of the organic solvent. In general, control of vacuum for vesicles by infusion was more problematic than for vesicles by evaporation, and the comments regarding vacuum made for vesicles by evaporation above and in Materials and Methods apply to infusion with vacuum at lower temperatures.

As described for vesicles by evaporation above, the method for producing vesicles by infusion requires additional dialysis or chromatography to remove residual organic solvent. In addition, the nature of the method suggests that solubilizer impurities may concentrate in the membrane and thus effect its physical properties (138). Finally, if vesicle phospholipid concentrations greater than 15 mM are desired, a concentration step must be included, with its requisite time, large buffer volumes (vacuum dialysis) and possible sample loss (Amicon concentration).

e. Vesicles by Detergent Dialysis

Vesicles by detergent dialysis are formed when phospholipid molecules dispersed in detergent micelles reorder into bylayers as detergent is removed from the system. Of the number of methods available for producing these vesicles, most well characterized are methods based on the slow

removal of nonionic detergents such as β-D-octylglucoside. If care is taken, these methods can be used to produce vesicles with diameters in the range of 56 to approximately 90 nm (187); sizes as large as 200 nm have been reported (181). The method was originally designed for use with mixtures of natural phospholipid and protein, but has been extended to include pure phospholipid systems, both natural and synthetic. A practical drawback of the most widely used of the methods is that it requires the long periods and large buffer volumes necessary for dialysis procedures. Recent modifications (181) have made use of adsorbant beads, which provide vesicles more rapidly but result in lipid losses of at least 30%. In experiments using these modified methods, a number of further conclusions have been drawn concerning the routine preparation of vesicles by detergent dialysis for physical studies.

First, Ueno *et al.* (187) have noted that the size of vesicles formed is a consequence of the kinetics of detergent removal; careful control of this step, therefore, is necessary if populations of the same diameter are desired from preparation to preparation. Also, difficulty in detergent removal has been noted. Reduction of the approximate ten-fold initial excess of detergent to an equimolar ratio takes place fairly rapidly; removal of remaining solubilizer proceeds much more slowly. These authors note that two sequential passes of a vesicle population (initial detergent-to-phospholipid ratio, 0.57) over a Sephadex G-200 column only reduces the ratio by about 40% (to 0.35). The calorimetric studies of Parente and Lentz (138) mentioned for vesicles by infusion above also considered vesicles by detergent dialysis, and arrived at the same conclusion: it was the presence of ≈30 mol% of detergent other contaminants that resulted in the less cooperative main transition.

f. Vesicles by Injection of Ethanol

Vesicles by injection of phospholipid in ethanol (VBI) were originally introduced by Batzri and Korn (12) for the production of vesicles of minimal diameter. Vesicles are formed by the

association of phospholipid molecules as solvent dissolves and disperses upon inje
aqueous buffer. Kremer *et al.* (103) observed a dependence of vesicle size on the conce
(in ethanol) of injected phospholipid, and so extended the method to the production of ve
large diameters. In a slightly modified procedure, VBI produced by slow injection
characterized using gel filtration chromatography and ^{31}P NMR in a study of the rel
between vesicle diameter and phospholipid asymmetry (see Thompson *et al.*, ref. 131).

In reviewing methods available for producing unilamellar vesicles, Szoka and Papahadjopoulas (181) describe the VBI method as "simple, rapid and gentle." Kremer (103) reports that vesicle diameter and number of lamellae are unaffected by most preparative conditions; only phospholipid concentration in injecting ethanol is critical (see above and Figure 3). Unresolved is the question of injection speed; Kremer reports decreasing polydispersity with increasing rates of injection with no significant effect on vesicle size. Others (131) suggest that only slow injections produce large-diameter vesicles. In either case it is interesting to note that the smallest vesicles available by this technique are on the order of 22 nm, the diameter of VBS.

The method to produce VBI can be applied to both natural and synthetic phospholipids without loss of lipid. The insolubility of phospholipids with acidic headgroups can be overcome using the free acid form of the lipid or by using a small percentage of water in the injecting ethanol. (See the section on phospholipids at the beginning of this discussion). By virtue of the general denaturing effect of ethanol, it would seem unlikely, however, to be of general use in the reconstitution of membrane protein. Because of its miscibility with water, it can be argued that the tendency of solubilizer impurities to be concentrated in the membrane would not be as great in the methods to produce vesicles by evaporation and by detergent dialysis; these two presumptions have not been tested. Two related, potential shortcomings are the low relative concentration of phospholipid and the significant concentration of ethanol in the final vesicle volume.

For the standard method, the concentration of vesicles produced is ≈ 7.5 mM, with a final concentration of ethanol in buffer of 5% (v/v). It has been suggested that higher final concentrations of ethanol be avoided, as they result in increased polydispersity (assessed by light scattering, ref. 103). Nordlund *et al.* (131), however, report that concentrations up to 30% (v/v) have little effect on vesicle characteristics, and suggest that populations with reproducible characteristics can be produced by a sequence involving injection, removal of ethanol, concentration and high-speed centrifugation.

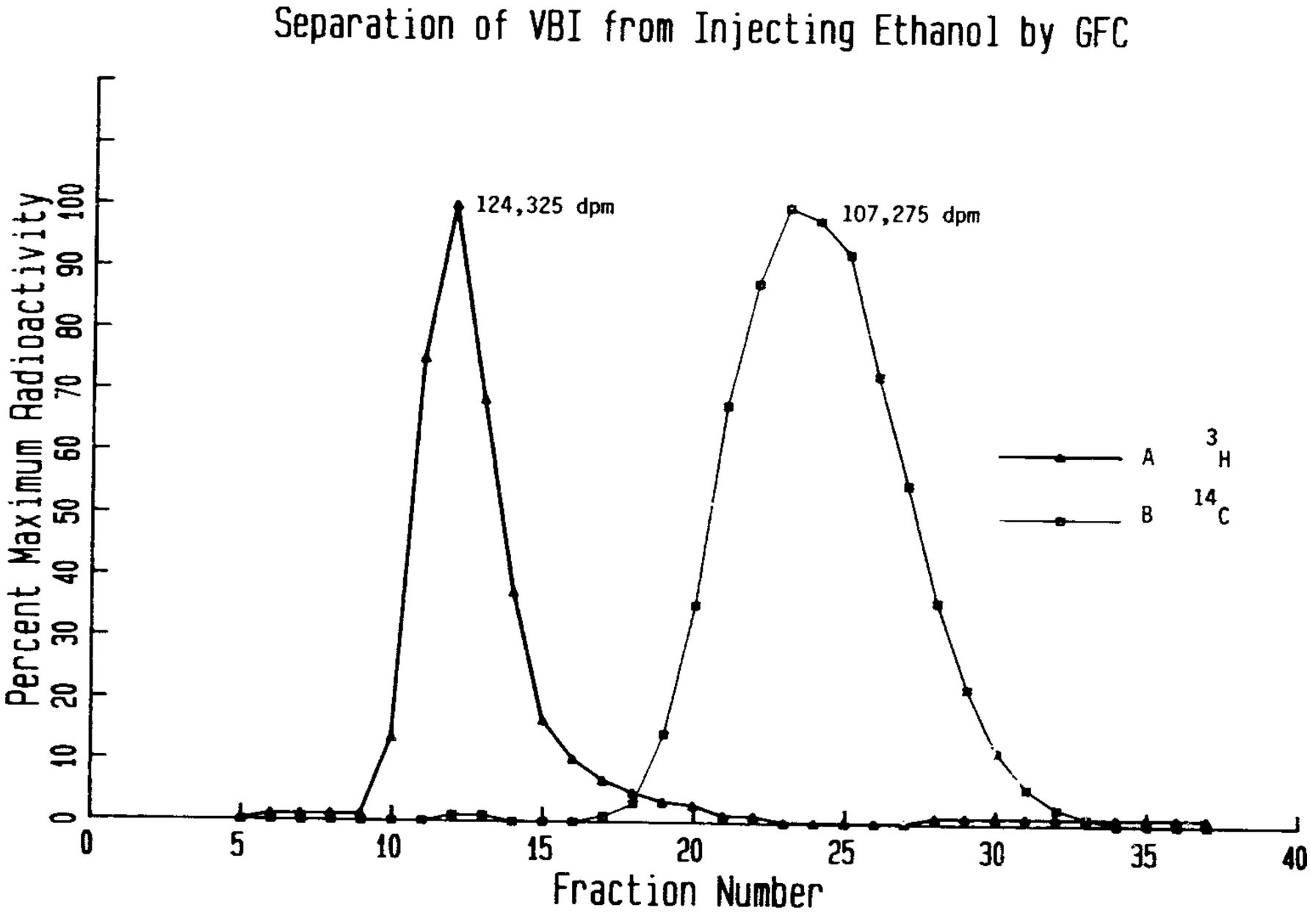
B. Assessing Chemical Purity, Diameter and Dispersity of Vesicles

1. Purity and Diameter

It was the VBI method, then, that was chosen as a system to produce large diameter vesicles, based on the relative merits of purity, efficiency of production, reproducibilty and applicability to a variety of phospholipid systems. The series of procedures used to produce each population for physical study was that of Nordlund *et al.* described above. A number of studies were performed to determine appropriate conditions for each step, then to characterize the vesicles produced in terms of freedom from solubilizer, vesicle diameter and homogeneity. Figure 4 presents data from the chromatography of a ^{3}H-labeled preparation of DMPC in ^{14}C-labeled ethanol; solvent is quantitatively removed by a single rapid pass over this gel filtration material. Residual ethanol is less than 0.2% of original, corresponding to an approximate 0.01 µl per 500 µl fraction. Subsequent passages were deemed unnecessary.

Vesicles were then characterized with respect to size and dispersity (homogeneity with respect to diameter and number of lamellae). As noted in the introduction, available methods for size

Figure 4 Separation of VBI from Injecting Ethanol by Gel Filtration Chromatography (GFC). VBI of ^{3}H-DMPC were prepared according to the standard method, except that the concentration of PC in ethanol was 40 mM, and the injecting ethanol was ^{14}C-labeled. Buffer was 10 mM PIPES, 0.36 mM $PrCl_3$, 0.2 mM EGTA pH 6.0. The post-injection vesicle suspension (in buffer/ethanol, 3:1) was chromatographed on Sephadex G25 as described in Methods. Elution was with an isotonic buffer containing NaCl and no $PrCl_3$. Levels of ^{3}H (A, PC) and ^{14}C (B, ethanol) in each fraction were determined by scintillation counting. See Materials and Methods for further details.


Separation of VBI from Injecting Ethanol by GFC
Percent Maximum Radioactivity
0
10
20
30
40
50
60
70
80
90
100
124,325 dpm
107,275 dpm
A
3H
B
14C
0
5
10
15
20
25
30
35
40
Fraction Number

characterization include techniques in microscopy, light-scattering, continuous-flow centrifugation and gel filtration chromatography. Only the latter two of these allow simultaneous size determination and fractionation of vesicles, and only gel filtration methods can be performed without need of special equipment. Sephacryl S1000 is the gel chromatographic material best suited to particle size analysis for particles with diameters from ≈10 to 300 nm. Ueno, *et al.* (187) write that the chromatographic procedure using Sephacryl S1000 "provides a more objective measure of average size than can be obtained by electron microscopy, and is especially advantageous for the determination of sample heterogeneity because a single elution profile contains information about all of the lipid present in the sample."

As the first step in using Sephacryl S1000 for characterization of vesicles, it was necessary to estimate or calculate the void (V_o) and total (V_t) volumes characteristic of each of the columns used. The void volume (V_o) for S1000 suggested by the manufacturer is 35-40% of the total (V_t) of the column. The actual V_o can be determined by passage of heat-killed Seratia as described in Methods, although this procedure is only adequate for determining the V_o in the absence of SDS. Column characteristics such as V_t, and consequently gel bead diameter and pore size, vary with the presence and absence of detergent and for columns "saturated" and "unsaturated" with respect to non-specific lipid binding: the S1000 bed shrinks markedly in the presence of SDS, and again upon application of the phospholipid used to "saturate" non-specific sites. It is therefore necessary to determine values of V_o for both sets of conditions so that values of the gel filtration parameters (K_{ave}, a function of V_o, V_t and the elution volume, V_e), can be determined and compared.

At the time of the calibration of the S1000 columns used in this study, no clear method for the determination of V_o in the presence of SDS was available, so the values of V_o(unsaturated,+SDS) and V_o(saturated,-SDS) are estimated in accordance with published data (151) to be $0.42 \cdot V_t$ and

0.46•V_t, respectively. The value of V_t used (31.8 ml for the 0.9 cm x 50cm column) was based on calculation of column volume, rather than measurement of V_e of a small solute. Where calculated and measured values are reported, agreement is within 6% (153).

Several methods have been suggested for translating the gel filtration parameters, K_{ave} (or K_d) into meaningful information about vesicle diameter. Reers *et al.* (151) present calibration data for Sephacryl S1000 determined at 0°C and 20°C in a plot of K_{ave} *versus* diameter, D. While the 20°C data were apparently linear, the 0°C data clearly were not, and were reportedly best fit by a three exponent Taylor function. Ollivon *et al.* (134) present calibration data for S1000 as the selectivity curve, log D *versus* K_d, and note an expected linearity for values of K_d between 0.2 and 0.8. A linear region was observed for data obtained from analysis performed on a Toya-Soda TSK G6000PW HPLC column, while the more variable S1000 data was described as being equally well fit by a quadratic equation.

Reynolds *et al.* (153) apply the rigorous treatment of Ackers (3, 4) in describing the Stokes radius,

$$a = a_o + b_o \, \mathrm{erfc}^{-1}(\varepsilon) \qquad \varepsilon = \{1 - [(V_e - V_o) / (V_t - V_o)]\}$$

where ε, the partition coefficient of system, is defined by $(1 - K_{ave})$, and where K_{ave}, the equilibrium constant for particle distribution between gel pore and fluid phase, is defined as the ratio of V_e to V_t after V_o is subtracted from each. Plotting the diameter of each standard *versus* $\mathrm{erf}^{-1}(\varepsilon)$, the value of the inverse error function (2) of the partition coefficient of each standard, should result in a presentation of data that is characteristic of the gel and independent of column dimensions (153). When data from several S1000 columns are processed in this manner, these authors have shown their calibration curves to be colinear through a region of K_{ave} of approximately 0.5 to 2.0, yielding approximate values of $a_o = -37.5$ and $b_o = 186$.

The analysis of Reynolds *et al.* (153) was applied to data collected using hydrogenated egg yolk PC saturated S1000 columns and the technique described in Methods. Two standards of known size were successfully chromatographed on the 0.9 cm x 50 cm column, allowing the estimation of the Stokes' radius (diameter) of populations of vesicles likewise chromatographed. Here we report the estimation of the diameter of a preparation of VBI based on its elution relative to a homogenous population of VBS and to carboxylated latex microspheres of defined diameter (Figure 5). See Huang (82) for another example of vesicle diameter estimation based on two standards.

An homogenous population of VBS of egg yolk PC was produced by high speed centrifugation of cup-horn sonicated vesicles (11) as described in Methods; it is the consensus of the literature that such vesicles consistently have a mean diameter of 22 nm, and as such they are frequently used as a size standard in gel filtration chromatography of other vesicles (151). These vesicles eluted in a peak at V_e = 30.0 ml, corresponding to a K_{ave} of 0.093 (Table 7). A commercially available preparation of carboxylated latex microspheres of 190 nm diameter was chromatographed, with the elution volume and calculated K_{ave} indicated in the Table. Finally, elution data for VBI prepared by the injection of 30 mM egg yolk PC in ethanol is presented. As with the VBS, these vesicles had been centrifuged at high speed to produce a homogenous population of unilamellar vesicles. The value calculated for the diameter of the VBI chromatographed is noted in the Table and discussed below. Values of the Stokes' parameters, a_o and b_o, for the experimental relationship are approximately -10 and 340, respectively, which compare favorably with values reported in and estimated from published data (see above and refs. 153, 151).

These data suggest that the VBI routinely produced by the slow injection of 30 mM PC in ethanol, and processed as described in Materials and Methods, are a population of vesicles of mean

Figure 5 VBI Diameter by Gel Filtration Chromatography (GFC) of Vesicles and Standards on Sephacryl S1000. Carboxylated latex microspheres (190 nm, A) and a homogenous population of vesicles prepared by sonication composed of ^{3}H-egg yolk PC (VBS, 21.5 nm, C) were chromatographed as diameter standards on a 0.9 cm x 50 cm column of Sephacryl S1000 (prepared as described in Methods). An homogenous population of vesicles prepared by injection composed of ^{3}H-egg yolk PC (VBI, B) was produced according to the standard method and was likewise chromatographed. Elution of microspheres and vesicles was followed by measurement of absorbance and by scintillation counting, respectively. See Methods for sample preparation and other details.

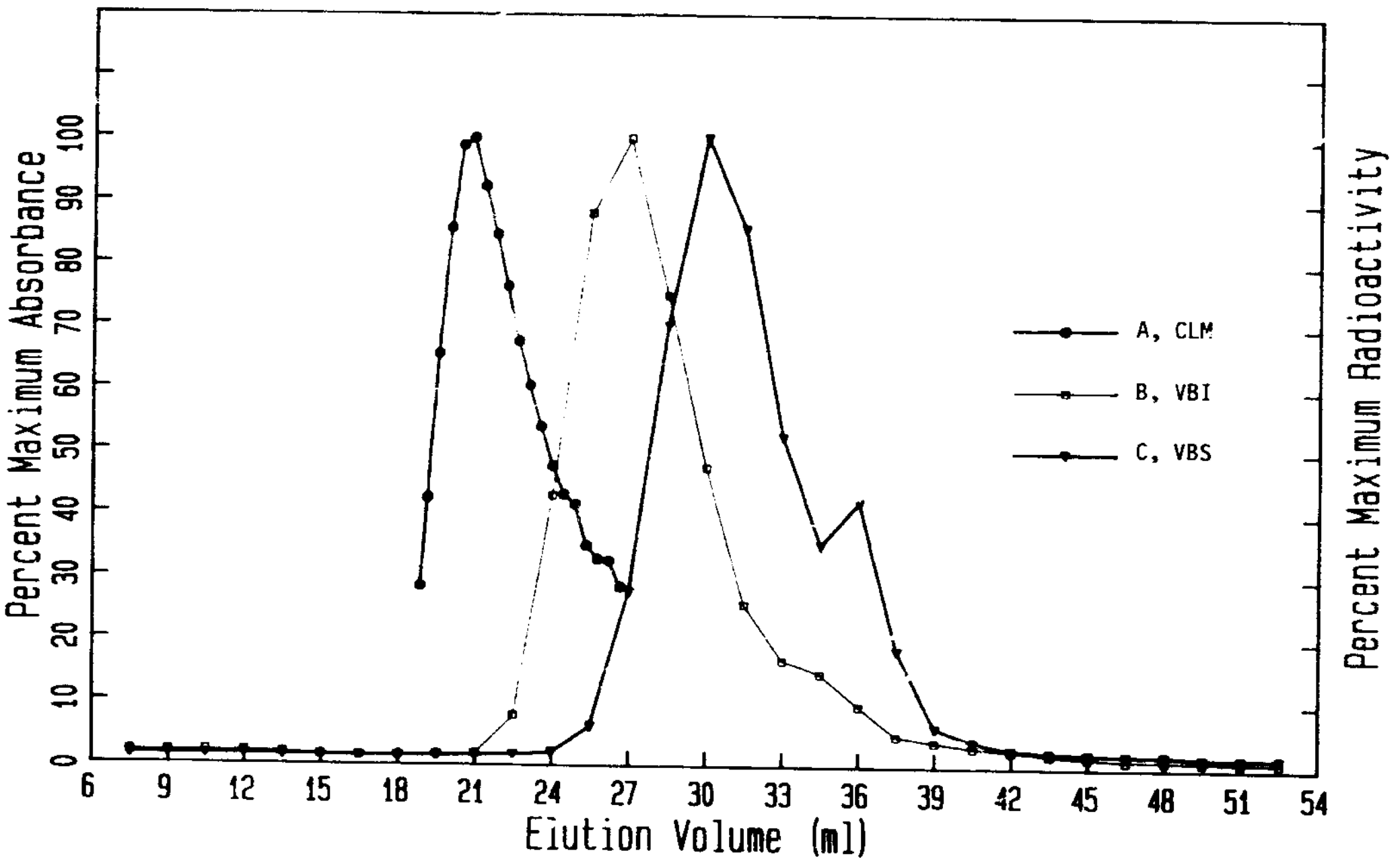

Sephacryl S1000 GFC of Standards and Vesicles
Percent Maximum Absorbance
Percent Maximum Radioactivity
Elution Volume (ml)
A, CLM
B, VBI
C, VBS
0
10
20
30
40
50
60
70
80
90
100
6
9
12
15
18
21
24
27
30
33
36
39
42
45
48
51
54

Table 7 Estimation of Vesicle Diameter by S1000 Sephacryl Gel Filtration Chromatography.[a]

	Sample[b]	Elution volume	K_{ave}[c]	erfc-1 (ε)[d]	Diameter[e] (nm)
A	Carboxylated latex microspheres (CLM)	20.9	0.41	0.586	190 ± 10
B	Vesicles by injection (VBI)	26.4	0.69	0.286	88 ± 7
C	Vesicles by Sonication (VBS)	30.0	0.90	0.093	22 ± 5

[a]Analysis is of experimental the data presented in Figure 5 and described in the text.

[b]Letter designations correspond to labels in Figure 5; volume abbreviations are defined in the text.

[c]The equilibrium constant for the distribution of particles between the fluid phase and the pores of the gel filtration medium, K_{ave}, is defined as:

$$K_{ave} = 1 - \varepsilon = (V_e - V_o) / (V_t - V_o)$$

See the text for the values of V_e, V_o and V_t used in K_{ave} calculations.

[d]The variable ε is defined in the preceding footnote. Values of erfc-1 (ε), the inverse error function, were taken from tables in Dwight (51). See Abramowitz and Stegum (2) for a description of the function, and Ackers and colleagues (3, 4) for an explanation of its application to gel filtration see Ackers and colleagues (3, 4)

[e]Diameter values are established values for standards (CLM and VBS) and the calculated value for VBI, based on the standard curve. See text for further details, including an explanation of indicated deviation.

hydrodynamic diameter of 88 nm. This conclusion is inconsistent with diameter values based on less rigorous methods of data analysis; a diameter of 53 nm, for instance, is derived when data are plotted as a selectivity curve as described above (134). It is, however, entirely consistent with the corresponding upper and lower limits of diameter suggested for this injection concentration and procedure, which are 80 and 100 nm, respectively (see ref. 103 and Figure 3). The maximum error in the diameter of the VBS standard iss estimated to be ≈5 nm. Error in the diameter of carboxylated latex microspheres is reported by the manufacturer to be ≈10 nm. Taken together these suggest precision within 10% for this determination.

2. Dispersity with Respect to Diameter

The same methods applied to determine vesicle size were used to assess the heterogeneity (with respect to size) of vesicles in standard preparations before and after a high speed centrifugation step (see Methods, Barenholtz *et al.*, ref. 10, and Lentz *et al.*, ref. 111). Figure 6 presents the results of Sephacryl S1000 gel filtration chromatography of ^{3}H egg yolk PC VBS before and after centrifugation. See Table 8 for tabulations based on this data. In both cases, phospholipid elutes as a single peak at an elution volume, $V_e \approx 30$ ml, with a shoulder at 36 ml. The 30 ml peak was judged to contain unilamellar vesicles based based on centrifugation and on ^{31}P NMR characteristics in the presence of a shift reagent (data not shown).

The latter peak appears at a position corresponding to a diameter smaller than the theoretical minimum, when a variety of vesicle preparations (DISP, VBS, VBI) are chromatographed; it is unaffected by centrifugation and extrusion procedures. It is conjectured to be a sloughing-off of phospholipid in a vesicular form, prompted by the passing band of applied vesicles. This raises in-

Figure 6 Homogeneity of VBS: Gel Filtration Chromatography (GFC) on Sephacryl S1000. Samples of VBS composed of ^{3}H-egg yolk PC were produced according to the standard method and were chromatographed prior to (A) and following (B) the high speed centrifugation step. Vesicle elution was followed by scintillation counting. See Methods for further details.

Vesicle Homogeneity by Sephacryl S1000 GFC : VBS

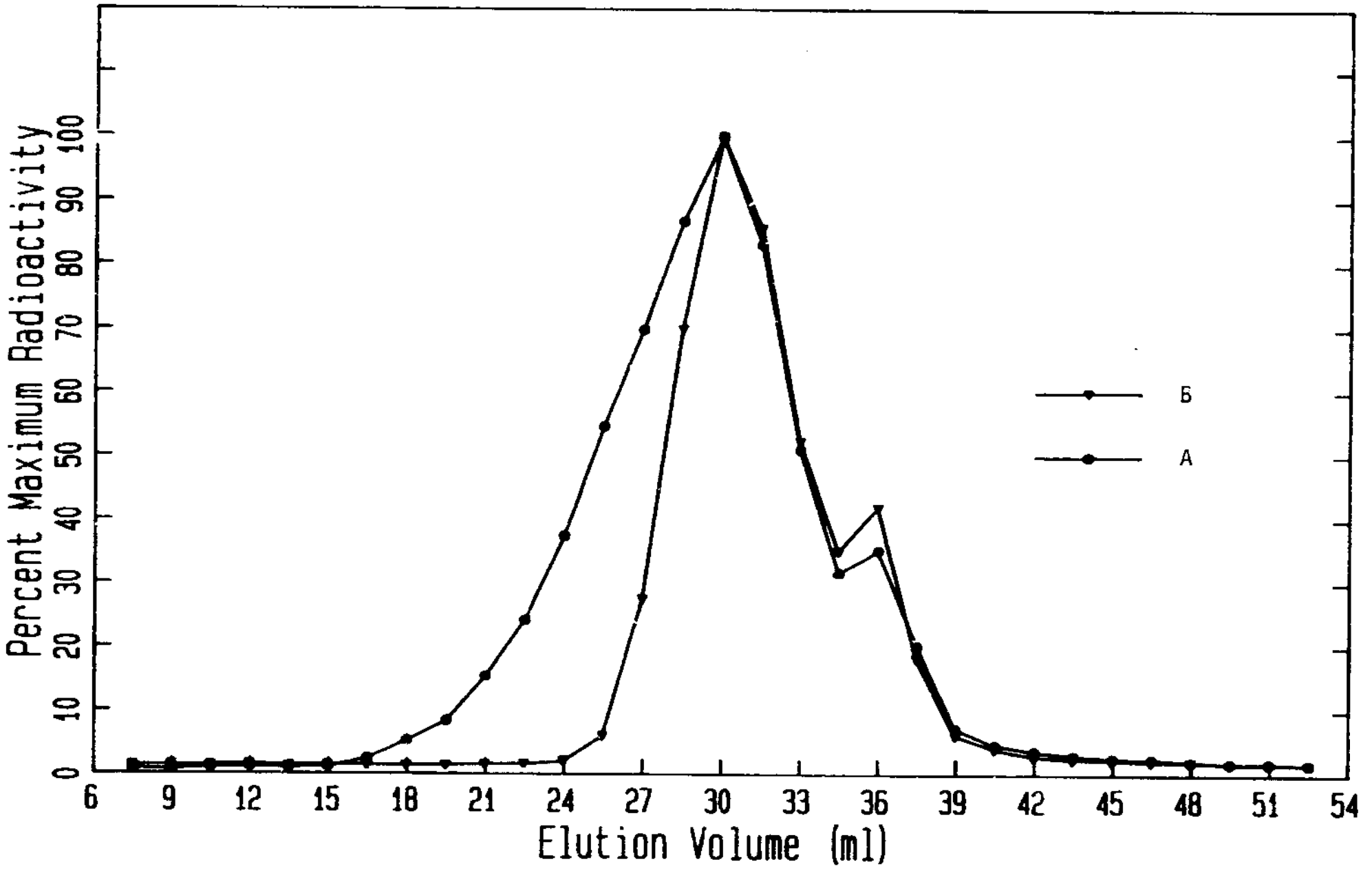


Table 8 Characteristics of Centrifuged and Uncentrifuged Preparations of VBS and VBI by Gel Filtration Chromatography.[a]

Chromatography System: Vesicle Preparation	Elution Volume, V_e (ml)	$V_{1/2}$ (ml)[b]	Hold-up (mol %)[c]	Yield (mol%)[d]
Sephacryl S1000:				
VBS, uncentrifuged	30.0	8.7	6	100
VBS, centrifuged	30.5	5.4	10	75
VBI, uncentrifuged	31.7	7.4	0	100
VBI, centrifuged	31.4	5.6	5	98
TSK 6000PW (HPLC):[e]				
VBS, uncentrifuged	7.32	1.02	obs	100
VBS, centrifuged	7.54	0.82	0	80

[a]Sample preparations, centrifugations and chromatographic separations are as described in Materials and Methods. Data tabled here is derived from data presented in Figures 6, 7 and 8.

[b]Peak width at half height, in units of milliliters.

[c]Vesicle hold-up, presumably due to interaction with gel: estimated mol % of phospholipid in fraction of profile with $K_{ave} \approx 1$. Abbreviation: *obs* - observable but not quantifiable.

[d]Vesicle yield after high speed centrifugation: estimated mol % of phospholipid remaining in region III (see text or ref. 10) after high speed centrifugation.

teresting questions regarding the nature of the interaction between eluting vesicles and the hydrogenated egg yolk PC-coated gel. Phospholipid mixing between the two pools was not tested here, but is reported to be insignificant (153). A similar shoulder was observed in NBD-labeled PC VBS when analyzed by HPLC using a TSK G6000 PW gel filtration column (Figure 7).

There is a measurable but arguably insignificant shift in the V_e of VBS after centrifugation; moreover, there is a narrowing of ≈40% in the peak width at half-height. The same is observed in the HPLC experiments on the TSK column mentioned above, where the decrease is approximately 20%. In both cases, the narrowing appears to be primarily attributable to a decrease in concentration of vesicles eluting nearer the void volume, V_o. This is interpreted as implying a narrowing of the size distribution around 22 nm, because of large diameter vesicles or aggregates pelleted by the centrifugation. Yield of the centrifugation step is on the order of 75-80%. Hold-up (depletion) of vesicles by the column appears to be on the order of 10% for S1000 and is negligible for the TSK column.

A comparable experimental characterization of labeled VBI produced by the standard method are presented in Figure 8. Here an initial V_e of 31.7 ml is observed, and there is again a small though measurable shift after centrifugation. Here, the shift to smaller V_e appears to correlate with a narrowing of ≈24% in the size distribution around 88 nm, somewhat curiously indicating a loss from both leading and trailing edges. Moreover, it is interesting to note that the homogeneity of VBI is initially greater than the homogeneity of VBS, but it is comparable after centrifugation. The high yield and the apparent increase in the homogeneity of centrifuged vesicle populations encouraged continued use of this one hour preparative step.

Figure 7 Homogeneity of VBS: Gel Filtration Chromatography (GFC) on TSK G6000 PW.
VBS of egg yolk PC labeled with fluorescent probe N-NBD-PE were produced according to the standard method and were chromatographed on this high performance column prior to (A) and following (B) high speed centrifugation. Vesicle elution was followed by flow-through fluorometric detection of the N-NBD label. For further details see Materials and Methods.

Vesicle Homogeneity by TSK G6000 PW HPLC/GFC

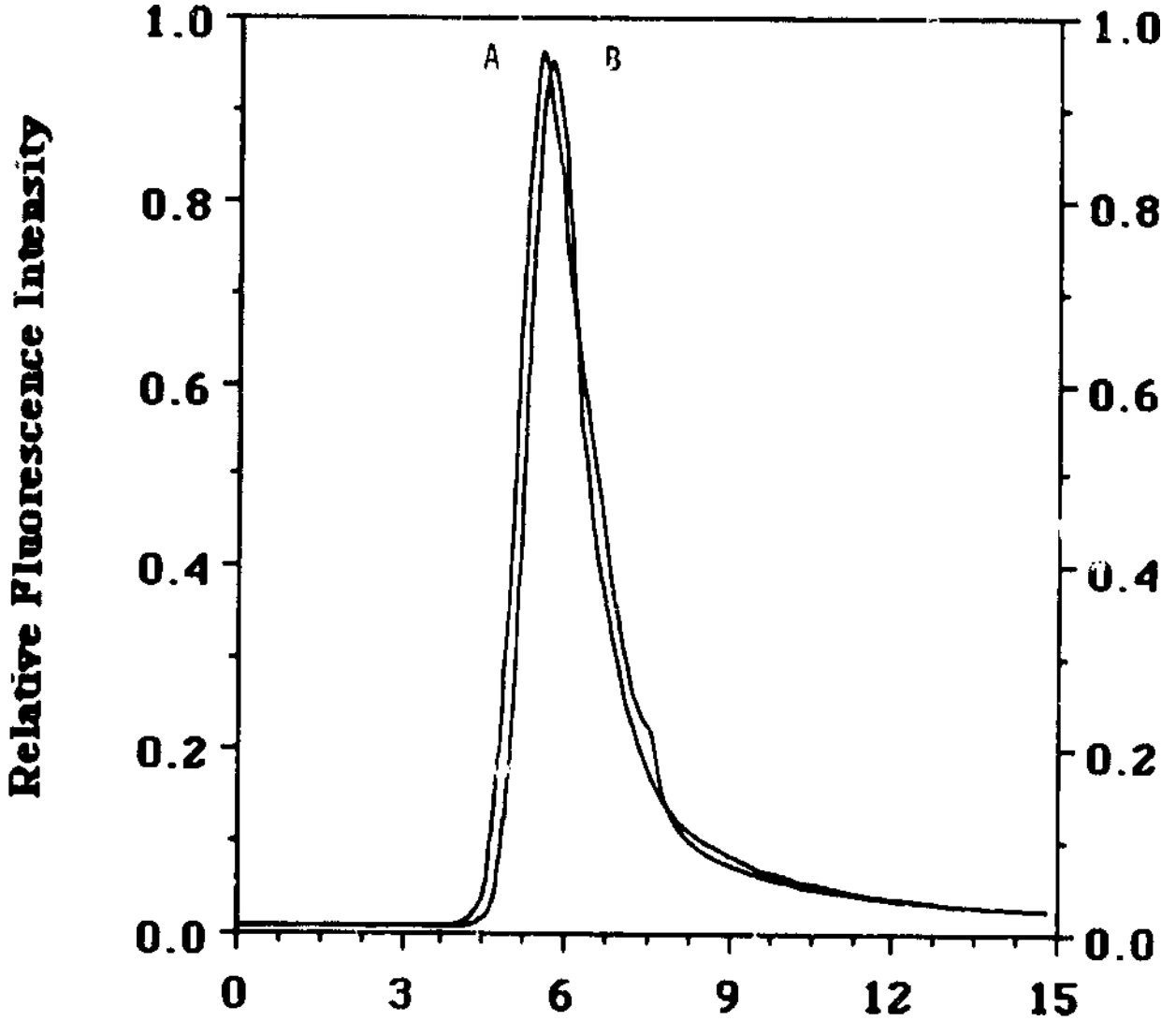
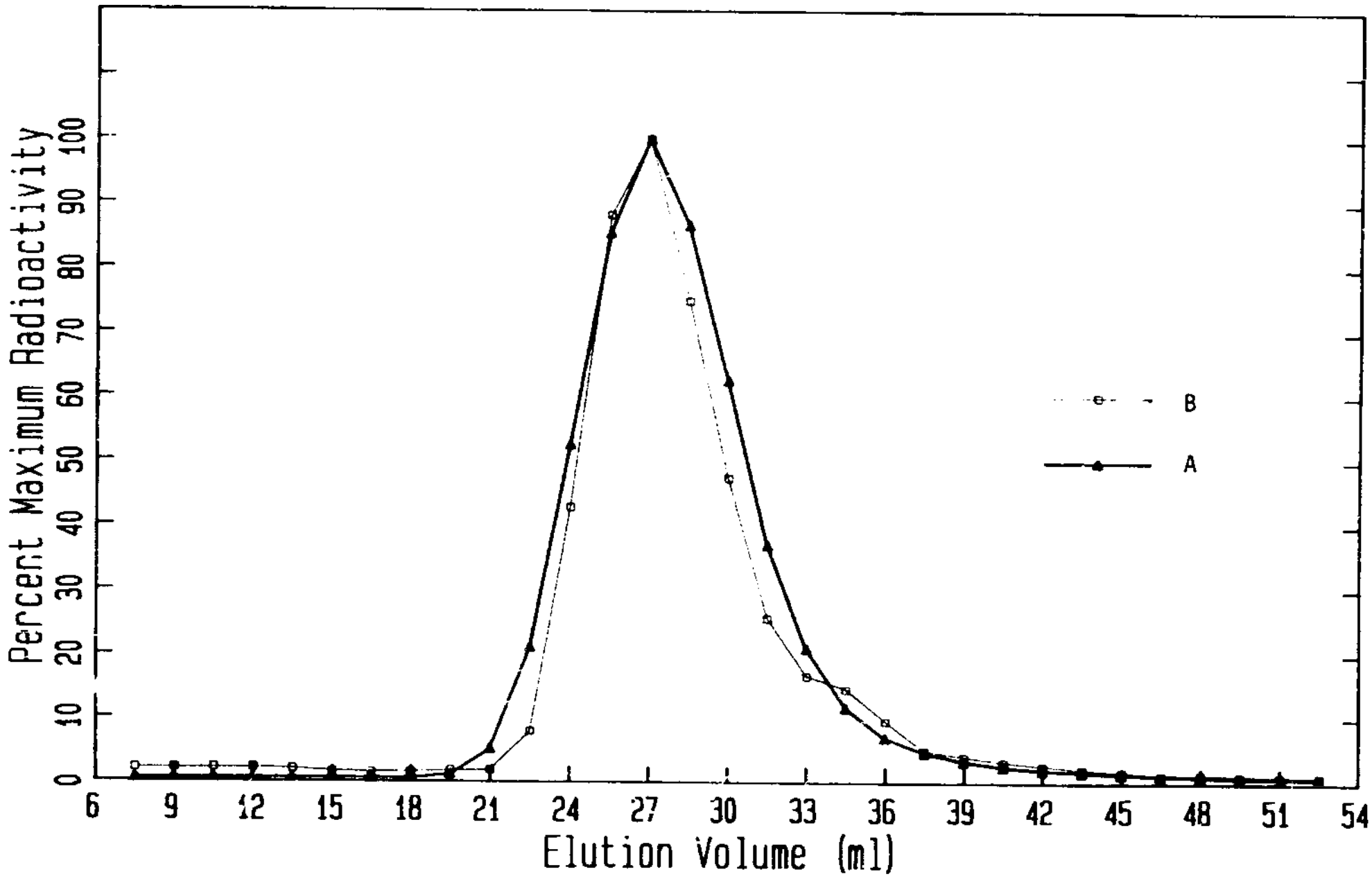


Figure 8 Homogeneity of VBI: Gel Filtration Chromatography (GFC) on Sephacryl S1000. Samples of VBI composed of ^{3}H-egg yolk PC were produced according to the standard method and were chromatographed prior to (A) and following (B) high speed centrifugation. Vesicle elution was followed by scintillation counting. See Materials and Methods for procedural details.

Vesicle Homogeneity by Sephacryl S1000 GFC : VBI



C. Purification of Phospholipase D

1. General

At the time this work was initiated, the only activity of phospholipase D (PHLD) that was well characterized was that isolated from Savoy cabbage, *Brassica bullata*. While commercial preparations of the enzyme were available, the noted presence of other lipases and variability from lot to lot (ref. 31 and present work) suggested purifying the material from the biological source. A lack of success in reproducing the overall method of Allgyer and Wells (ref. 5, based in part on the method of Yang, ref. 207) led to the development of the standard method by Dr. Robert Clancy in this laboratory (31, 32). The Allgyer report indicates purification to homogeneity; the Clancy procedure produces an enzyme preparation free of contaminating lipases, where PHLD was 5-20% (w/w) of the total protein, with specific phosphohydrolase activity of 1 - 1.3 units (μmol min^{-1}). While methodological differences could account for the different results obtained in the two labs, there is reason to question the degree of homogeneity reported by Allgyer and Wells (25, 5). In any case, the Clancy purification scheme provided a means to obtain a relatively pure enzyme preparation, as well as a basis for varying scale and for future attempts to improve the purification.

Changes in scale required the modifications noted in Methods. Other modifications were simply based on experience with stability of the enzyme during homogenization and resuspension steps. The extreme seasonal variation in quality (and periodic unavailability) of the starting material led to the use of the commercial preparation of enzyme noted in Materials, Methods and in figure legends, and characterized with the Clancy preparations below. At times, material was so dark with pigment that the diethyl ether extraction of Allgyer and Wells (5, 31) was reintroduced to allow normal spectrophotometric procedures.

2. Gel Filtration and Hydrophobic Affinity Chromatography

One further attempt was made to improve the yield and purification of the Sephadex G200 gel filtration step; the result was no better than previously reported (data not shown; ref. 31). The apparent difficulty is the amount of time required for the enzyme to remain on the column due to the high viscosity of the eluent [50% EG (v/v) in buffer] and consequent low flow rates. As noted, the use of ethylene glycol to stabilize to protein requires ambient temperature for reasonable flow rates (31); both room temperature operation and the slower flow rates that result from operation at 4°C result in the appreciable loss of activity. Simple batch experiments preclude an explanation based on rapid denaturation due to interaction with the resin. Results in other laboratories have indicated that the use of medium pressure (and therefore higher flow rate) bio-compatible chromatographic materials and systems can, in some cases, alleviate such problems.

A gel filtration purification step was attempted using a less compressible cross-linked agarose, since this type of polymer matrix allows higher operating pressures and flow rates. Biogel P-150 (BioRad) was chosen based on the reported molecular weight range of PHLD (5, 31) and on the reported fractionation range of the gel. A sample from the acetone precipitate fraction of the purification process was applied to a column as described in Methods. All substances with significant absorbance at 280 nm eluted as a single peak at or near the void volume of the column (data not shown). This data and the observed shrinkage of the P-150 gel upon equilibration with TES-EG buffer suggest that the actual pore size of the gel is somewhat smaller than reported for strict aqueous conditions, resulting in the exclusion of PHLD and other proteins. Flow rates allowed by this gel did prove to be better than those of the corresponding Sephadex, and an attempt to fractionate the acetone precipitated preparation on Biogel P-200 might be profitable.

The required desalting of the enzyme following ammonium sulfate precipitation suggested the application of hydrophobic interaction chromatography, where proteins applied at high salt are eluted in a gradient of decreasing salt according to apparent hydrophobicity. Preliminary batch experiments using a phenyl-substituted chromatographic material resulted in a small purification of the protein, with very little loss in activity (data not shown). If this result could be extended to a column purification with gradient elution, the enzyme could be purified and stored as the stable salted-out form, and further purified as needed using the hydrophobic and affinity columns. Both the phenyl- and the γ-aminopropane-substituted materials are commercially available.

3. SDS Poly(acrylamide) Gel Electrophoresis of Purified Enzyme Preparations

As noted, the affinity-purified PHLD has a specific activity of 1.0 - 1.3 μmol min^{-1} mg^{-1} protein, in accord with values suggested by Clancy (31). A photograph of a Coomassie brilliant blue stained 7.5% SDS poly(acrylamide) gel electrophoresis experiment is shown in Figure 9. The standard curve used to calculate approximate molecular weights of the major bands is shown in Figure 10. Clancy reports five major polypeptides at this stage in the purification, with the 100 kD PHLD comprising 5-20% of the mixture; this is essentially what is observed in Figure 9. There has been some variability in the reported molecular weight of the enzyme (100-120 kD, refs. 5, 31). In addition, the presence of bands of ≈ 50 kD in SDS-poly(acrylamide) gels of the *100* kD peak of enzyme purified using the gel filtration chromatographic step of Allgyer and Wells (5) has been noted (31). These observations, taken with the clustering of bands at 50 and 100 kD seen here, support the suggestion that a protease is at work (31), and that inhibitors might be a useful addition to buffers during purification.

Figure 9 SDS-PAGE of Preparations of PHLD: Estimation of Enzyme and Contaminant Molecular Weights. A 10% gel analysis was performed on standards with the molecular weights noted (A); and on 30 μg affinity-purified PHLD (B). Numbers 1 - 8 identify significant bands in B and assign molecular weights based on the selectivity curve (Figure 10) calculated from the migration of standards in A. See the text and Methods for further details.

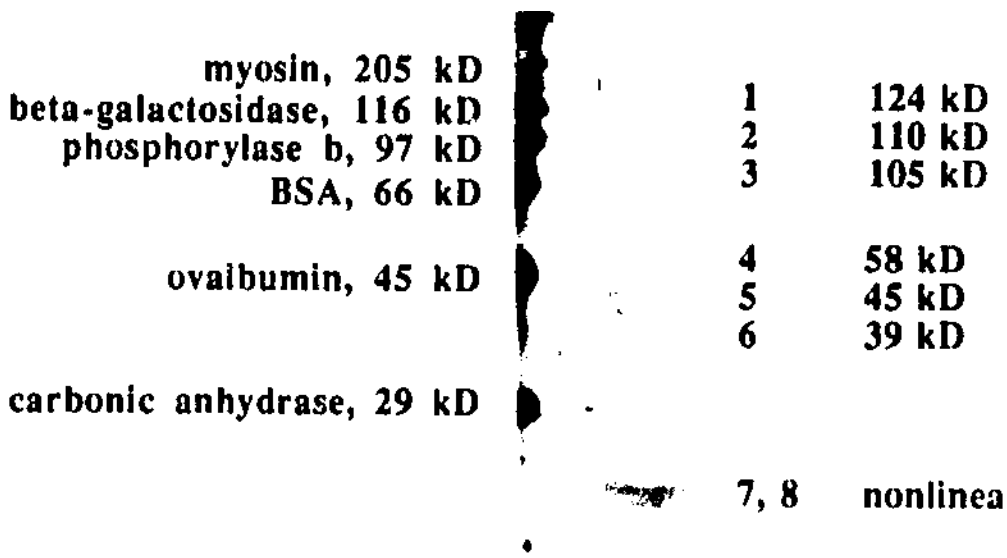
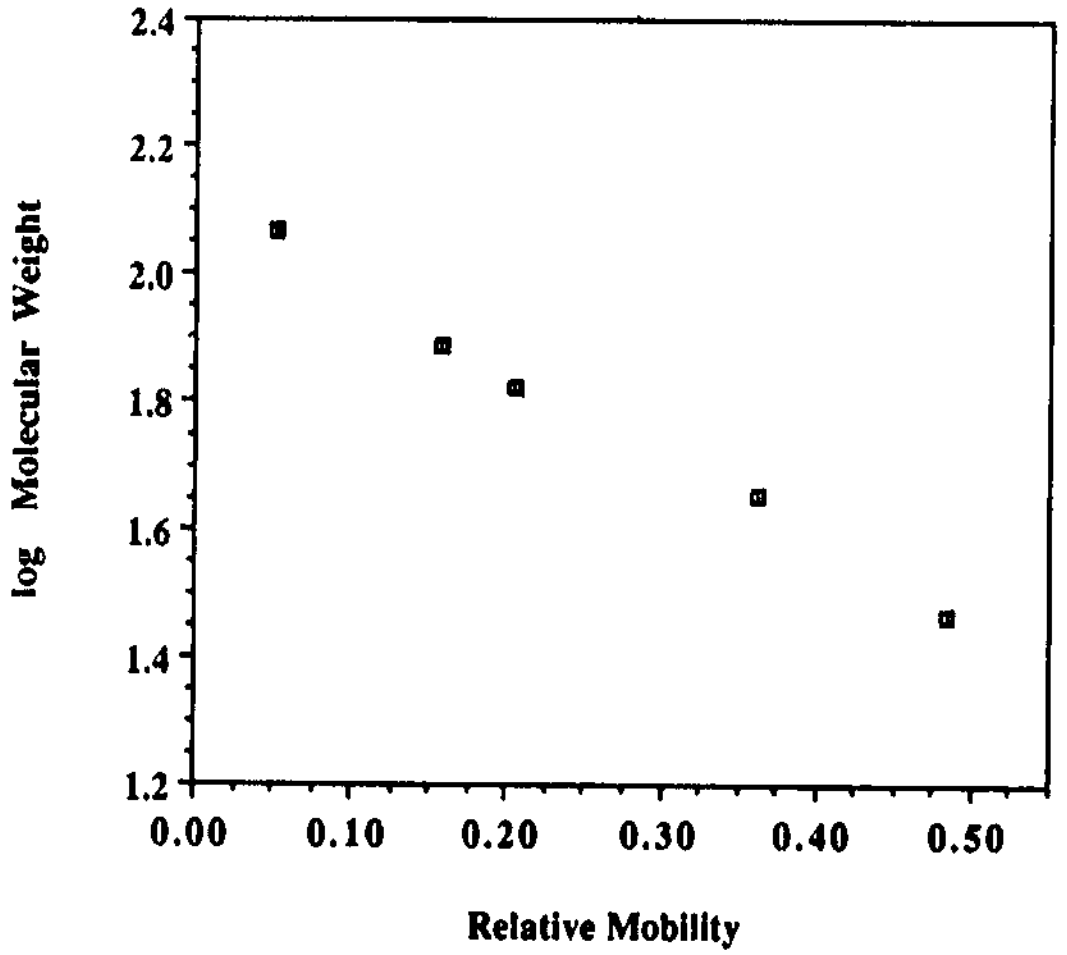

myosin, 205 kD
beta-galactosidase, 116 kD
phosphorylase b, 97 kD
BSA, 66 kD
ovalbumin, 45 kD
carbonic anhydrase, 29 kD
1 124 kD
2 110 kD
3 105 kD
4 58 kD
5 45 kD
6 39 kD
7, 8 nonlinear
A B

Figure 10 SDS-PAGE of Preparations of PHLD: Selectivity Curve. The mobility of each molecular weight standard on a 10% gel (Figure 9) is plotted as a function of the log of the molecular weight; see the preceding figure and the text for details.

Estimation of PHLD Molecular Weight by SDS PAGE



The higher percentage SDS-poly(acrylamide) gel electrophoretic experiment presented in Figure 11 was performed in order to confirm the apparent relative purity of commercial preparations of the enzyme (based on specific activity) and to investigate the possibility of contamination by low molecular weight proteins. Shown are standards and the affinity-purified PHLD described for the previous gel figure, samples of commercial PHLD, and two stocks of PHLD acetone precipitate. The PHLD band in each lane runs just above the top border of the portion of the gel shown. Lanes for the affinity-purified and the acetone precipitate from which it is purified share a number of bands which are of equal intensity or greater intensity in the purer material. PHLD and the low molecular weight bands may be co-purifying through the final affinity column; alternatively, these bands could be the result of continuing proteolysis. The apparent purity of the commercial enzyme (lane C), judged by the lack of visible contaminating bands at equal gel loadings relative to the other enzyme sources, is striking. The use of this preparation for preliminary studies and at times when Savoy cabbage was unavailable was encouraged by this result and by its high specific activity (≈1 unit mg^{-1}).

Affinity purified material was eluted and stored in a buffered solution that is 50% (v/v) ethylene glycol, TES-EG. The buffer was changed on a routine basis to produce a working stock of enzyme in a comparable buffer free of ethylene glycol. The buffer change was performed by dialysis (5), or by gel filtration followed by either Amicon concentration (31) or vacuum concentration with dialysis using the Prodicon (described in Methods). All were generally performed in the presence 0.3 M *myo*-inositol . The results of a simple experiment showing the inhibitory effect of the presence of residual ethylene glycol on the phosphohydrolase activity of the enzyme, and testing the viability of performing the ethylene glycol removal in the absence of inositol, is shown in Figure 12. The activity of affinity purified enzyme determined using the Clancy assay is presented for enzyme after Sephadex G25 removal of >90% of the EG containing buffer and compared to samples further dialyzed and concentrated.

Figure 11 SDS-PAGE of Preparations of PHLD: Evaluation of Enzyme Purity. A 15% gel analysis of standards with the molecular weights noted (A); affinity-purified PHLD (B); commercial PHLD from Sigma Chemical (C); acetone-precipitated PHLD (D and E). Sample loadings in lanes B and C were 30 μg protein and in Lanes D and E were 20 μg protein. PHLD (≈105 Kd) would be expected to run just above the top border of the portion of the gel shown. See the preceding two figures and the text for details.

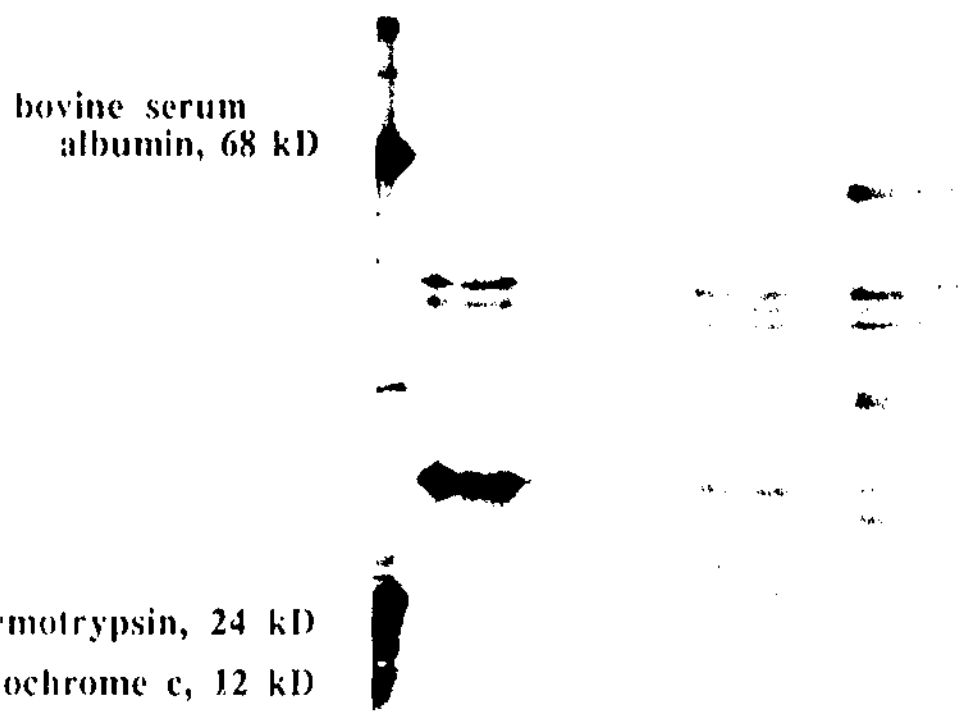
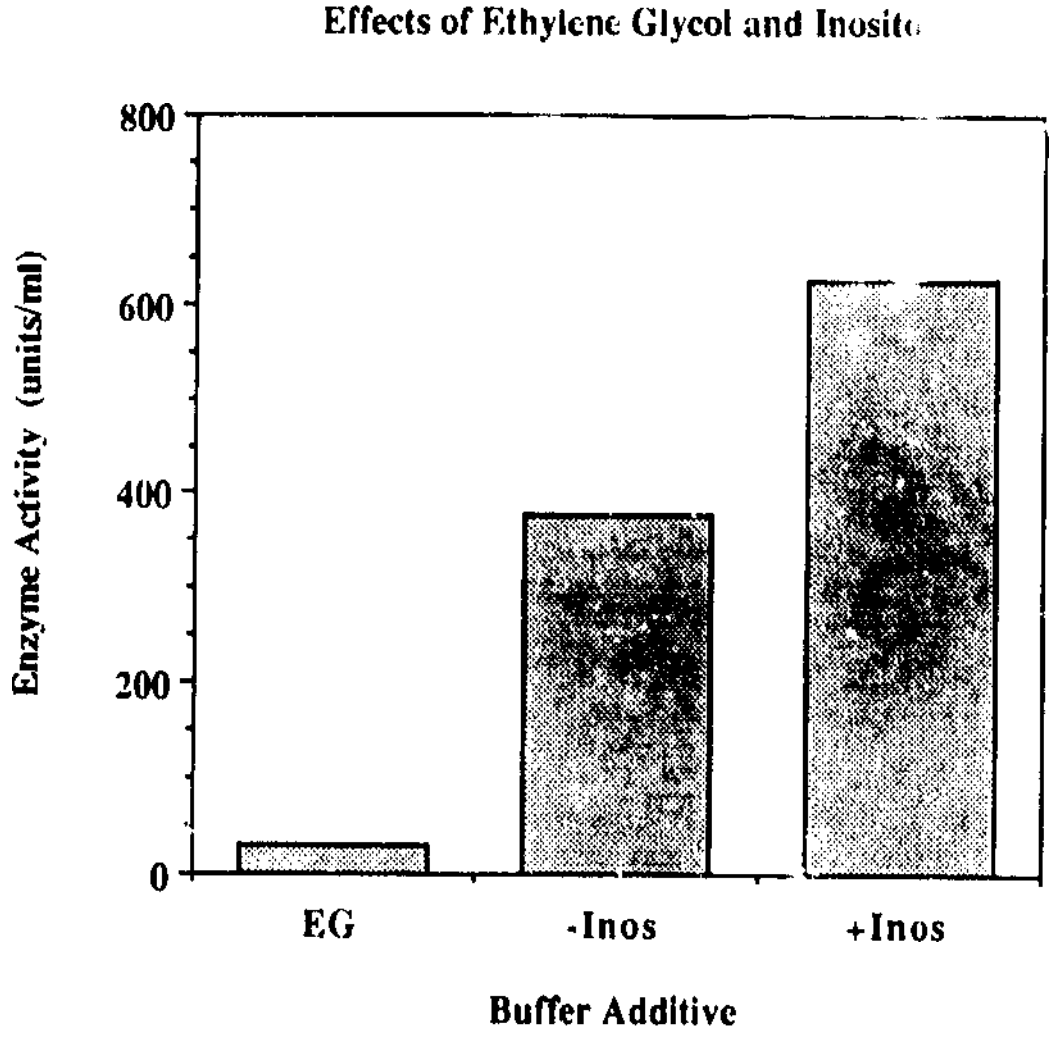

bovine serum
albumin, 68 kD
chymotrypsin, 24 kD
cytochrome c, 12 kD
A
B
C
D
E

Figure 12 PHLD Catalyzed Hydrolysis of PC in a Two-Phase Assay System: Effects of Ethylene Glycol and Inositol. The activity of affinity purified PHLD was determined using [choline, N-C^3H_3]-labeled substrate and the buffer/diethyl ether assay of Clancy (31, 32). The amount of ^3H-choline liberated was determined by scintilation counting of an aliquot of the aqueous phase after centrifugation to separate the phases. Compared are the activities of equal amounts of protein in buffer (5 mM TES pH 7.0) with residual (< 5%) ethylene glycol, assayed before (EG) and after vacuum dialysis to remove the residual glycol. Vacuum dialysis was carried out against buffer containing (+Inos) and free of (-Inos) 0.3 M *myo*-inositol. See the text and Materials and Methods for further details.


Effects of Ethylene Glycol and Inosit
Enzyme Activity (units/ml)
800
600
400
200
0
EG
-Inos
+Inos
Buffer Additive

There are large increases in activity with the removal of ethylene glycol, both when inositol is absent (≈15-fold) and present (≈24-fold) in the dialysate.[1] The stability afforded by the presence of inositol in the dialysis buffer is significant (≈1.6-fold), though reasonable activity is maintained in its absence. Both ethylene glycol and inositol alter the hydrogen bond structure of water, and could therefore affect the structure of PC at the interface of aqueous and phospholipid phases; moreover, both are potential substrates for the alcohol transferase activity of PHLD. It was therefore decided to further vacuum concentrate and dialyze after Sephadex gel filtration and to omit inositol from the dialysate in hope of simplifying the interpretation of enzyme-vesicle studies.

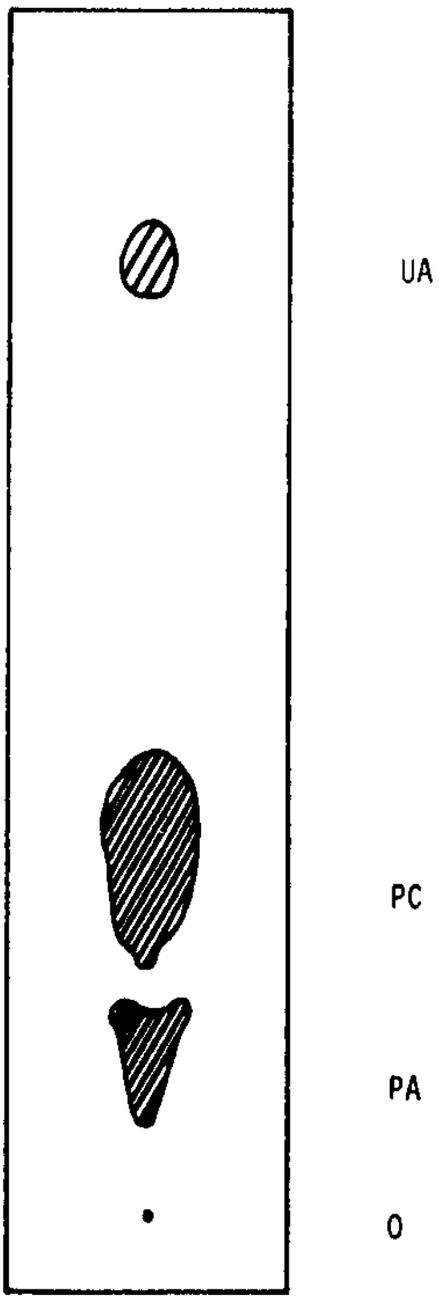
D. PHLD Conversion of PC to PA: Early Work

The results of an early experiment suggested that it might be possible to use PHLD to alter the headgroup composition of the outer leaflet PC of vesicles by sonication (VBS). The VBS were treated with commercial PHLD in the presence of Ca^{2+}, and the reaction product was centrifuged to pellet multilamellar structures. The significant fraction of PA found in the supernatent (apparent in the TLC separation of extracted phospholipids shown in Figure 13) strongly suggests that a population of intact, mixed PC/PA vesicles was the result of the digestion. Also present is an observable fraction of I_2-stained material migrating in the fatty acid/neutral lipid portion of the plate. A control extraction of the enzyme preparation yielded no lipid. These results are in agreement with earlier reports (31, 32, 55, 191) of a contaminating phospholipase A-type activity in commercial preparations of PHLD, making it necessary to further purify or to find an alternative

---

[1]The complicated system of potential substrates, activities and phases makes it impossible to do more than state this observation. It is possible, for instance, that one set of rates being measured is that of the alcohol transferase (transphosphatidylase, "base-exchange") reactions involving the exchange of an inositol or an ethylene glycol molecule for one of choline. Both would be detected, as the assay measures release of free choline, whether by hydrolysis or transphosphatidylation.

Figure 13 PHLD-Catalyzed Hydrolysis of PC Vesicles: TLC of the Lipid Extract of a VBS Reaction in the presence of Calcium. A homogeneous preparation of VBS of DMPC was prepared in 10 mM succinate, 10 mM $CaCl_2$ pH 6.0 according to the standard method. After two-fold dilution, the VBS were incubated for 60 min. at 37°C in the presence of ≈100 units/ml of commercial PHLD. The reaction mixture was centrifuged to pellet all but homogeneous VBS, 0.8 ml of the 7.3 ml supernatant was extracted by the method of Bligh and Dyer (19) and the extract was analyzed by TLC. See Materials andMethods for further procedural details. Non-standard abbreviations are Q - origin and UA - unknown A.


UA
PC
PA
O

source of the enzyme. Although no attempt was made to determine the yield of the preparation, the size of the lipid pellet from the post-reaction centrifugation was significant.

Further thought was then given to the conditions of the reaction and the possibility of hydrolysis in the absence of calcium. Choice of reaction conditions was influenced by the variety of reports available describing pH and calcium concentration optima and substrate specificity of cabbage PHLD. Many reports suggest that the calcium requirement of the enzyme is absolute (76). This would complicate matters, as VBS of PC and of PC/PA have been shown to be unstable with respect to aggregation and precipitation, vesicle size and lamellarity, and the asymmetry of the membrane (99, 116, 163, 206), especially in the presence Ca^{2+} (69, 101, 129, 133, 137, 152). These observations, in part, would be expected to hold true for VBI (129).

Others suggested that the calcium requirement of the enzyme varies with the "form" of the enzyme. Up to three forms of the enzyme have been reported, varying in molecular weight, thermolability and calcium requirement (5, 142). In the report of variation in calcium requirement (5), Allgyer and Wells note two pH optima for a homogeneous preparation of enzyme. The first is seen at 30 mM calcium at pH 5.6, the second is seen at 5 mM calcium at pH 7.25.

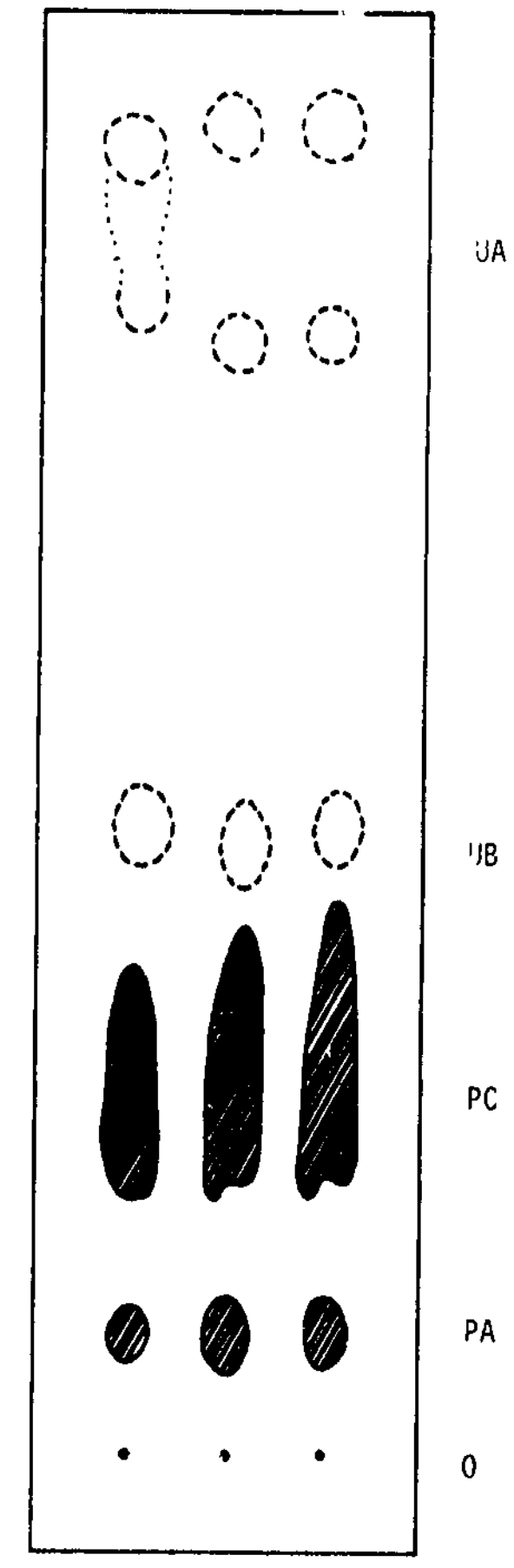
Thorough studies on PHLD by M. M. Rhakimov, Sh. R. Mad'yarov and coworkers (142-146) confirm and extend the earlier analysis of Dawson (33b). Both observe that enzymes which carry out reactions at interfaces are sensitive to any parameter which might alter the balance of forces acting at the interface (33b, 146, 193). Dawson note as important factors the physical nature of the substrate (whether monomer, micelle, etc.), the ratio of substrate mass to the area of the interface available for enzyme binding, the characteristics of the packing of substrate molecules, the electrostatic field at the interface and the presence of non-aqueous solvents. Rhakimov *et al.* specifically note that the calcium concentration optimum for the phosphohydrolase activity varies

with the chemical nature of the substrate, with its physical form and in the presence or absence of non-aqueous solvent, detergent and solid adsorbants. These authors conclude that the rate of the PHLD-catalyzed hydrolysis of PC is dependent upon "the curvature and charge of the interface, as well as the orientation of the phospholipids on the interface" (146). They further state that "activation by calcium is only an alternative means of imparting a catalytically active conformation," and that "enzymatic hydrolysis is possible in the absence of calcium ions."

The next set of studies, then, was carried out in an attempt to establish conditions for the Ca^{2+}-free hydrolysis of PC in the simplest available system: the Clancy assay, described above, using commercially available PHLD. The first approach was to consider pH values in the neighborhood of 7.25, where Allgyer and Wells (5) observed a second pH maxima in the presence of 5 mM calcium. Also, low enzyme concentrations and longer time courses were explored in order to avoid the aggregation of vesicles observed in high enzyme concentration experiments. Shown in Figure 14 is the result of a TLC analysis of phospholipids extracted by the Clancy method (31) from reaction mixtures at pH 7.0 and 7.5. PA is present, as are several contaminating fatty acid/neutral lipid species. The presence of the latter encouraged the analysis and precautions described and discussed at the opening of Methods and of this section.

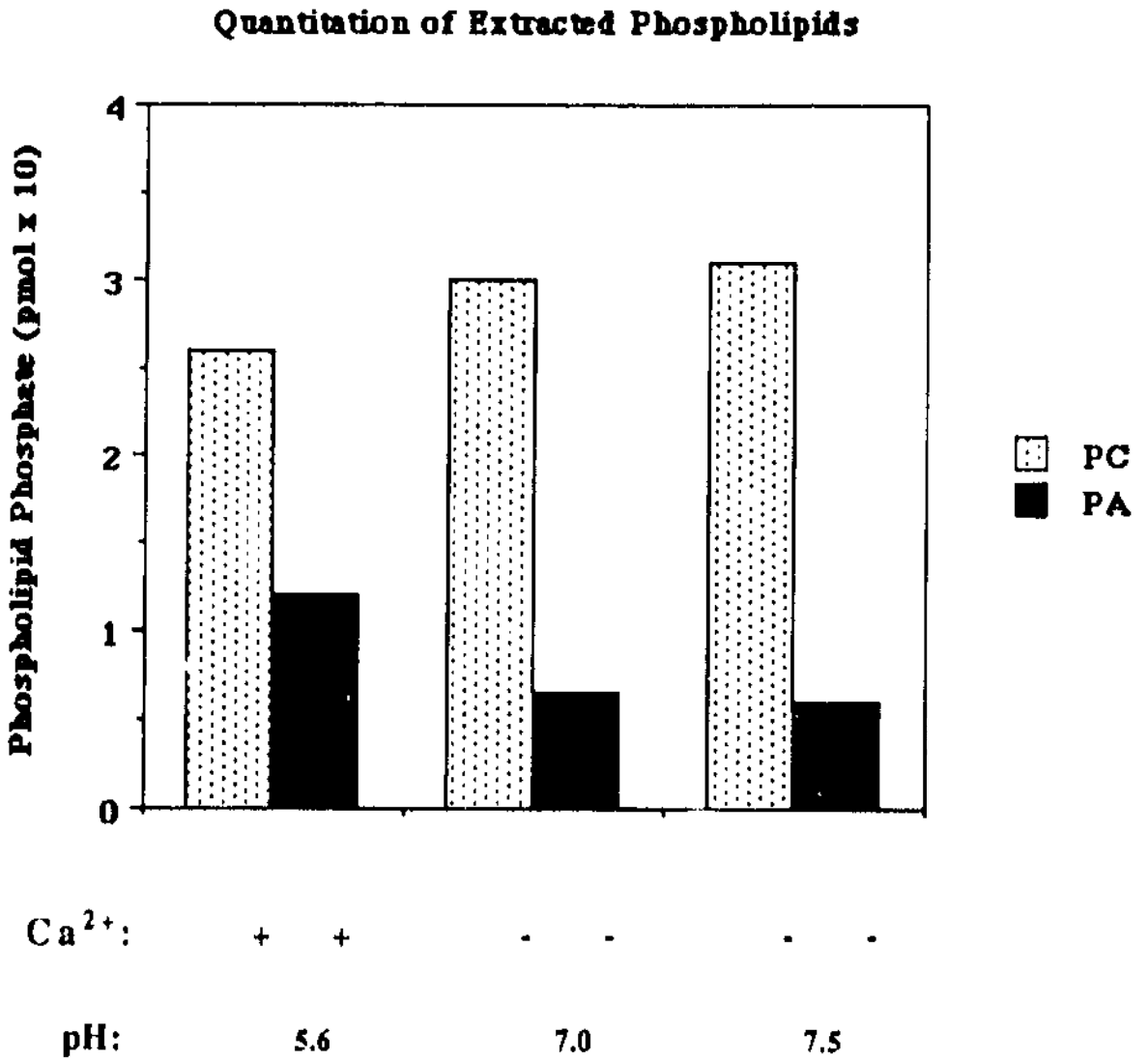
Spots on these plates were analyzed for radioactivity (PC) and for inorganic phosphate after ashing (PA). The absolute values of the quantitation are graphed in Figure 15. The control reaction at lower pH in the presence of calcium resulted in conversion of more than 30 mol% of initial PC to PA. At the higher pH, both calcium-free reactions yielded approximately half that fraction of PA. Hence, the yield was on the order of the amount required for reasonably accurate quantitation by NMR and significant enough to effect physical properties assessed by fluorescence methods.

Figure 14 PHLD-Catalyzed Hydrolysis of PC in a Two-Phase Assay System: TLC of the Lipid Extract of a Reaction in the Abscence of Calcium. Relative rates of hydrolysis of monolayers of ^{3}H egg yolk PC were determined under Clancy assay conditions, except that a calcium-free buffer (30 mM MES, 30 mM Tricine, 100 mM NaCl) was substituted for samples at pH 7.0 and 7.5, and the reaction duration was 6 hrs. Each assay mixture contained 6 units of commercial PHLD. Assay mixtures were extracted, and the extract was analyzed by TLC. Non-standard abbreviations are Q - origin, and UA and UB - unknowns A and B. See the legend to Figure 12 and the text for further details.



pH	5.6	7.0	7.5
Ca	+	-	-

Figure 15 PHLD-Catalyzed Hydrolysis of PC in a Two-Phase Assay System: Quantitation of PC and PA in Extract. PC and PA from the experiment described in the preceding figure were eluted from scraped silica and phospholipid concentration was determined for PA by phosphate analysis and for PC by scintillation counting. See Methods for further details.


Quantitation of Extracted Phospholipids
Phospholipid Phosphate (pmol x 10)
4
3
2
1
0
PC
PA
Ca2+: + + - - - -
pH: 5.6 7.0 7.5

E. PHLD Conversion of PC to PA: Vesicle Work

An early pH profile of a short hydrolysis using the calcium-free buffer and low enzyme concentration described in Figure 14 above is shown in Figure 16. This pattern of pH maxima at 5.5 and 6.5 was generally reproducible for DISP, but required phospholipid labeled to higher specific radioactivit to avoid occasional spurious data; this was especially true with reactions such as the 10 min. reaction shown here. Total PA measured was on the order of ≈10 mol% for the reaction at pH 5.5.

The changes from a fairly crude preparation to a relatively pure preparation of PHLD, and from DISP to vesicles by injection (VBI) as substrate result in changes in optimal reaction conditions. Figure 17 presents the results of 0.5 hr. experiment comparing the hydrolysis of DISP and VBI at pH 5 6 and pH 7.25. Maximum hydrolysis in the figure reflects conversion to about 14 mol% PA in total PC. Contrary to what was seen in Figure 16, commercial PHLD shows no hydrolytic activity in the absence of calcium at pH 5.6. When the substrate is changed to VBI, however, this form of the enzyme is reasonably active. Generally VBI are seen to be equal or better substrates than DISP for this enzyme preparation when conditions are otherwise equivalent.

As expected, activity in the presence of the calcium cation was always higher than the corresponding experiment performed in its absence. Somewhat unexpectedly, values at pH 7.25 in the presence of calcium are higher than those at pH 5.6. This activity might reflect the high pH/low calcium form of the activity mentioned above, though the reported calcium concentration corresponding to a pH optimum of 7.25 was 5 mM, *versus* the 25 mM here. Generally this enzyme preparation was most active at pH 7.25, except that the population of VBI at pH 5.6 in the absence of calcium was the better substrate compared to its pH 7.25 counterpart.

Figure 16 PHLD-Catalyzed Hydrolysis of PC Vesicles; Dispersions, Varying pH. Relative rates of hydrolysis of a standard preparation of DISP of ^{3}H egg yolk PC were determined using a calcium-free buffer (30 mM MES, 30 mM Tricine, 100 mM NaCl pH 5.0 - 7.5). Each reaction contained 6 units of commercial PHLD. Quantitation of released choline was by scintillation counting after extraction according to Bligh and Dyer (19). See the legend to Figure 12 and Methods for procedural details.

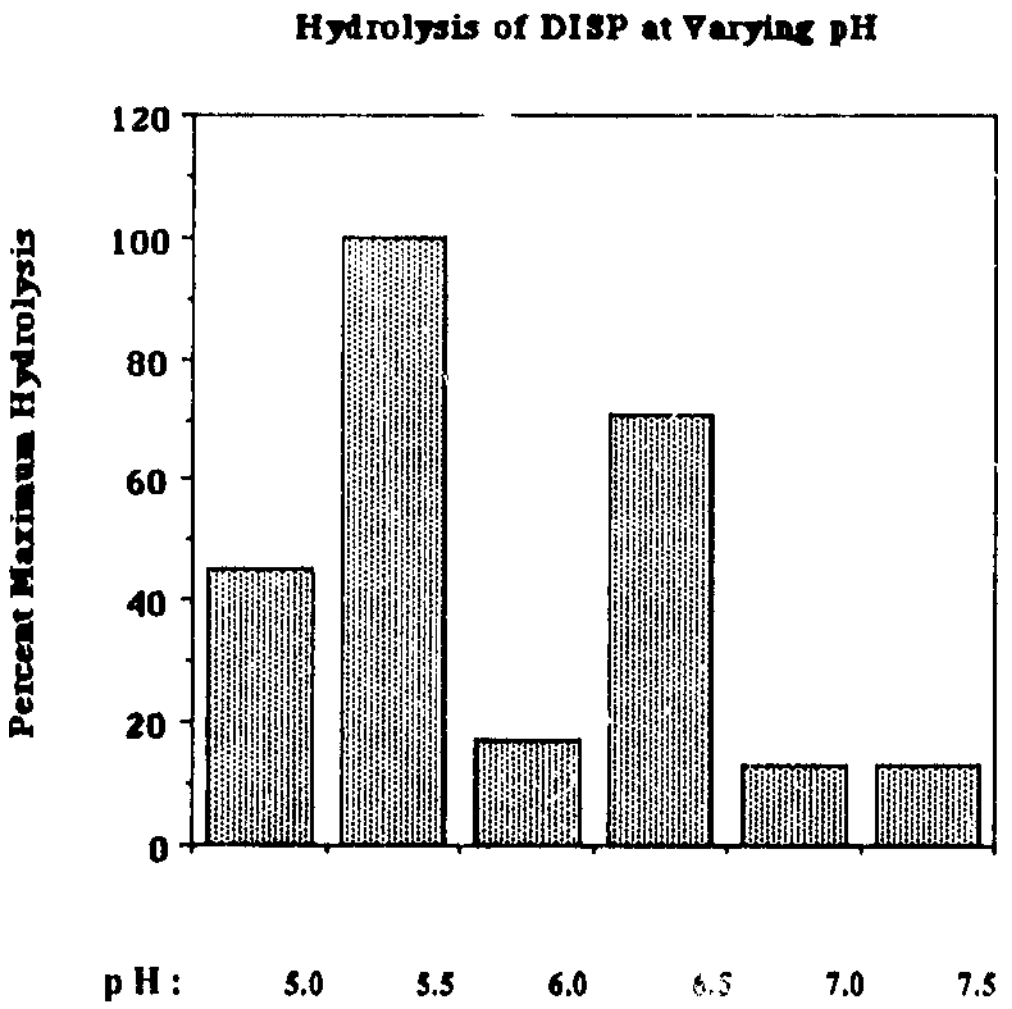
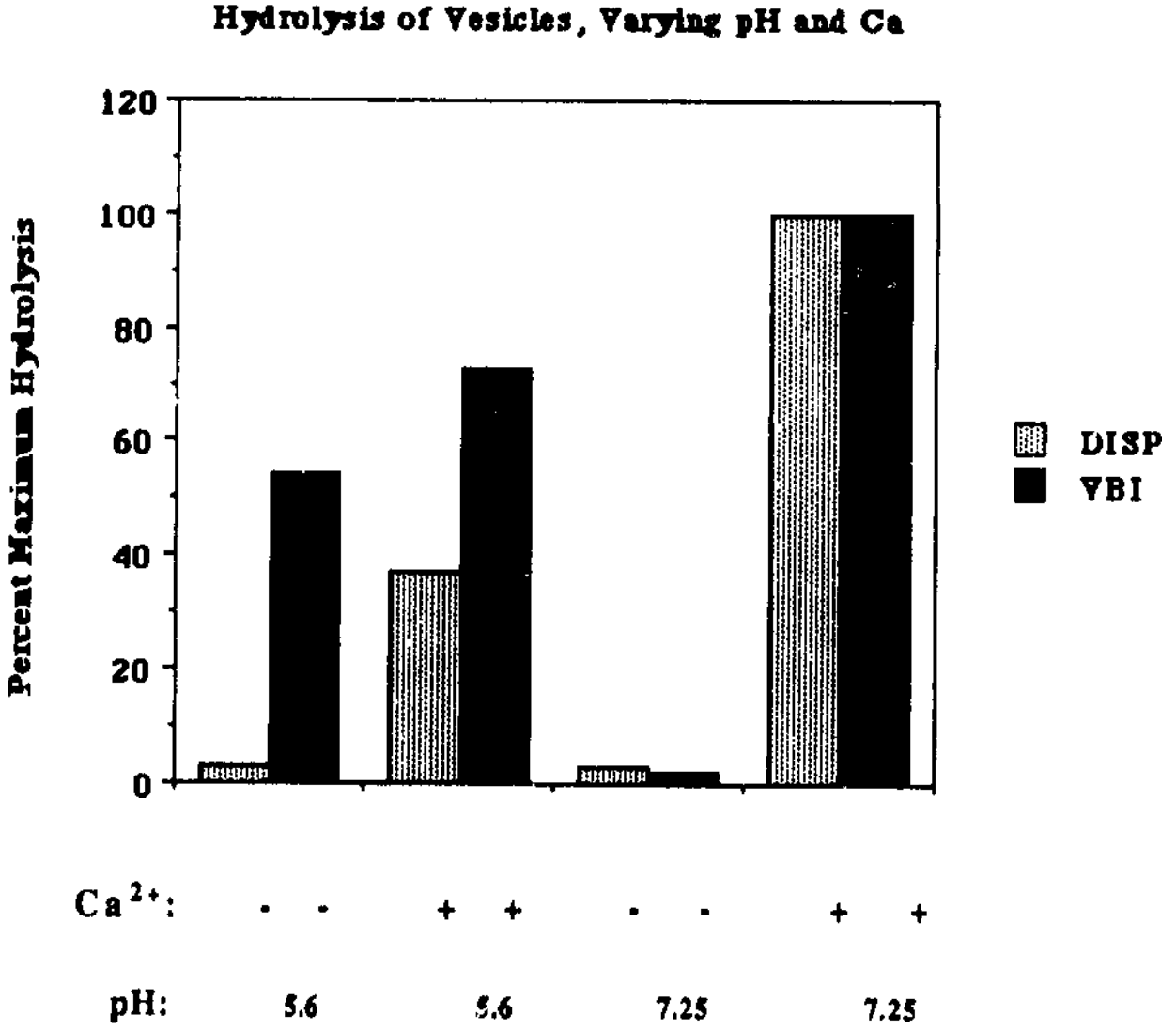

Hydrolysis of DISP at Varying pH
Percent Maximum Hydrolysis
120
100
80
60
40
20
0
pH :
5.0
5.5
6.0
6.5
7.0
7.5

Figure 17 PHLD-Catalyzed Hydrolysis of PC Vesicles: Vesicle Type, pH and Calcium Concentration Variations. Relative rates of hydrolysis of standard preparations of VBI and DISP of 3H egg yolk PC were determined at the indicated pH using the method described in the preceding figure; $CaCl_2$ (25 mM) was substituted for NaCl as noted. Each reaction contained approximately 16 units of affinity-purified PHLD. See preceding figures and Methods for further procedural details.


Hydrolysis of Vesicles, Varying pH and Ca
Percent Maximum Hydrolysis
120
100
80
60
40
20
0
DISP
VBI
Ca2+: - - + + - - + +
pH: 5.6 5.6 7.25 7.25

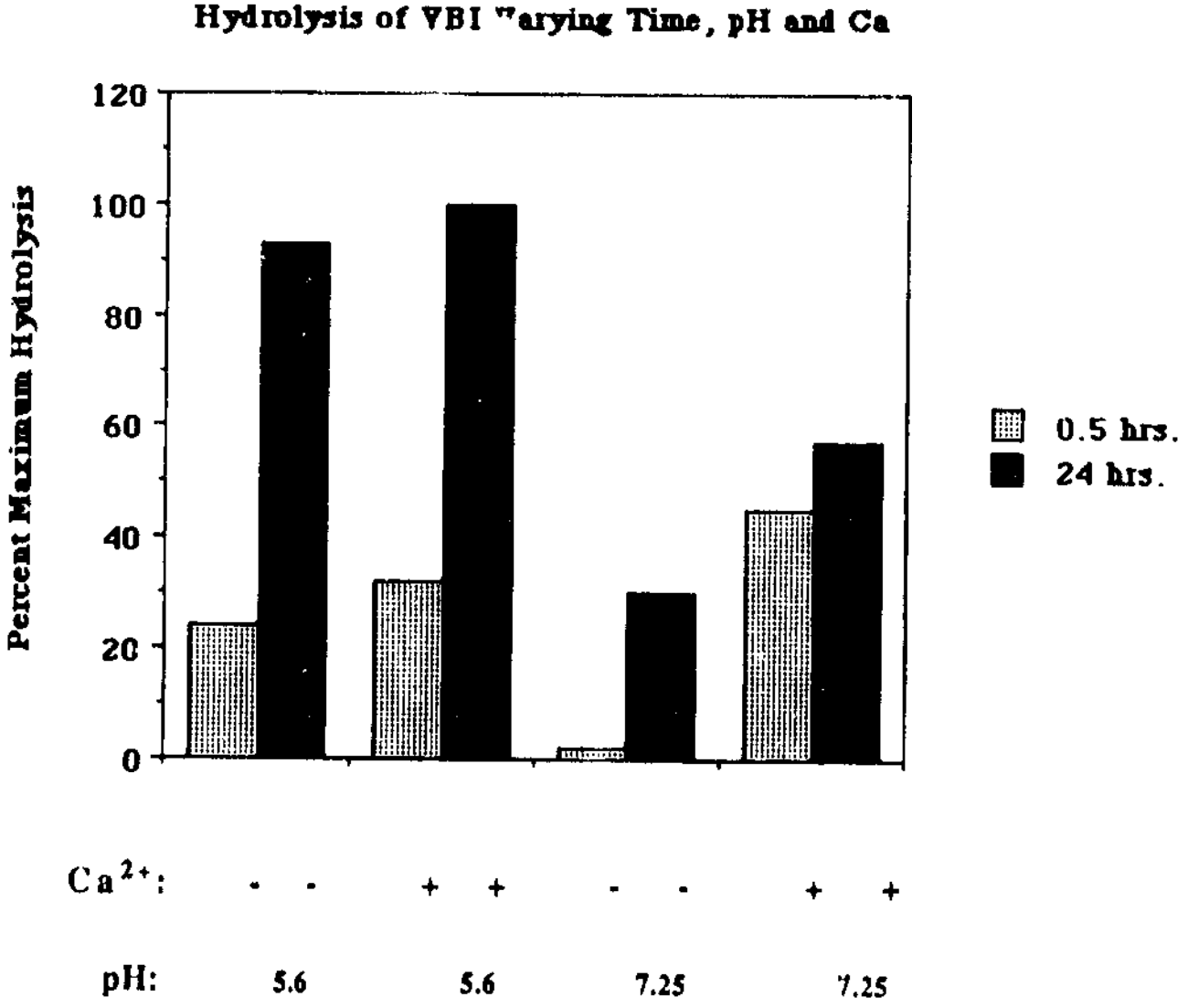
When the duration of the previous experiment with VBI was extended to 24 hr. and the concentration of calcium lowered to 5 mM, the results presented in Figure 18 are seen. Maximum hydrolysis is approximately 35 mol% PA in total PC. The 0.5 hr. reactions of VBI have yields which are essentially identical to those shown in the previous figure. The percent conversion generally increases with extended time, though in a manner disproportionate to the initial rapid rate. The exception is the pH 7.25 assay in the presence of calcium.

In this case, extending the time does not increase the yield; most available phospholipid is hydrolyzed in the first thirty minutes, and little more reacts (or becomes available) in the remaining hours of reaction. In both of the pH 5.6 assays, the initial yield is followed by continuing reaction. Both assays reach the final approximate yield mentioned above. This behavior probably reflects a degree of product inhibition by PA, perhaps greater at pH 7.25 that at 5.6. Alternatively, it could be taken to suggest that while a limited population of readily available substrate is presented to the enzyme by VBI at pH 7.25 in the presence of calcium, the available substrate at pH 5.6 is greater, perhaps by flip-flop of PA-PC as the concentration of PA builds in the outer leaflet.

In the process of performing these experiments, questions were raised concerning the ability of the two standard extractions to completely separate PA and PC from liberated choline. Quantitation of reaction rates and yields depends upon the ability to follow either the decrease in radioactivity in the PA + PC fraction, or the increase in radioactivity in the aqueous choline fraction. Any cross-contamination would make quantitation difficult. The extent of each reaction discussed above was determined by analyzing aliquots from one of the phases of an extraction system: ether or aqueous in the Clancy assay (31), and $CHCl_3/CH_3OH$ or H_2O/CH_3OH in the Bligh and Dyer assay (19).

One set of experiments demonstrated that both assays efficiently extracted both PA and PC into the organic phase if care was taken to lower the pH to protonate PA: the aqueous phase of each assay

Figure 18 PHLD-Catalyzed Hydrolysis of PC Vesicles: Varying Time. Relative rates of hydrolysis of standard preparations of VBI of ^{3}H egg yolk PC were determined as described in Figure 17, except that $CaCl_2$ and NaCl concentrations were 5 mM and 20 mM, respectively, and that reaction durations were as indicated. See the preceding figures for details.


Hydrolysis of VBI "arying Time, pH and Ca
Percent Maximum Hydrolysis
120
100
80
60
40
20
0
0.5 hrs.
24 hrs.
Ca2+: - - + + - - + +
pH: 5.6 5.6 7.25 7.25

was found to be free of phospholipid cross-contamination (data not shown). Results of the second experiment are shown in Table 9. Pairs of samples containing ^{3}H-choline were extracted in the manner described in Methods for the extraction of reaction mixtures. Volumes of each phase were measured, and aliquots of the aqueous phase were analyzed for radioactivity. Shown is the percent of initial choline present in the aqueous phase after extraction. It appears that choline is equally distributed between phases when extracted according to Bligh and Dyer, but is predominantly in the aqueous phase of the Clancy system. In the latter, residual choline can be removed from the organic phase by washing with an equal volume of distilled water (31).

Taken together, the data suggest that the phosphohydrolase activity is best monitored by following the change in (i) phospholipid species in the organic phase of either extract by TLC as described, or in (ii) choline concentration in the combined aqueous phase after Clancy extraction with washing. The first of these is tedious, requiring extraction, TLC, scraping and eluting of plates and scintillation counting or phosphate assays to quantitate phospholipid. The latter only indirectly gives access to the actual phospholipid concentrations present. These results prompted the design of an experiment to simultaneously determine the concentrations of both phospholipid species.

A PHLD-catalyzed reaction of VBI labeled nearly equimolar with ^{3}H-choline and ^{14}C-palmitoyl PC is shown in Figure 19. The slightly altered buffer and reaction conditions noted were changes necessary to optimize the reaction conditions for vesicles destined for analysis by ^{31}P NMR. There was no increase in turbidity during the course of the reaction, and no aggregation or precipitation was observed. The vesicles produced during this procedure were not further characterized.

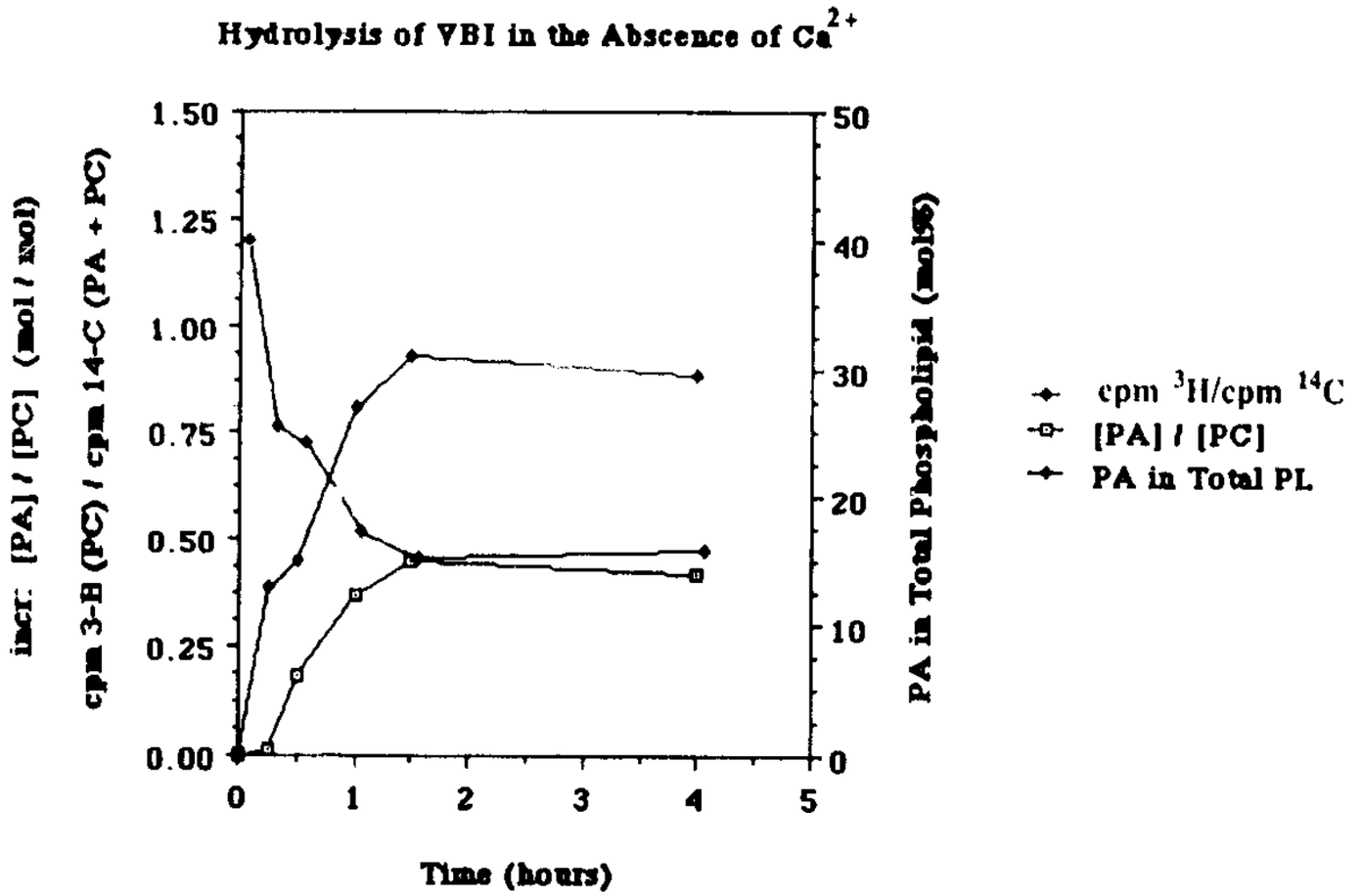
Table 9 Partitioning of Choline between Aqueous and Organic Phases during Standard Extractions.

	Extraction Method (ref.)[a]	Fraction of Initial Choline in Post extraction Aqueous Phase (percent ± std. dev.)[b]
A	Bligh and Dyer (19)	51.5 ± 1.3
B	Clancy (31)	91.5 ± 0.4

[a]Extractions were performed as described in Materials and Methods, except scaled as necessary. The reference listed is that of the original paper describing the method. The aqueous sample was 727 µl of 10 mM HEPES, 100 mM KCl pH 7.0 containing 3.7 x 10^7 dpm [N-methyl-^{3}H] choline chloride. 73 µl of 5N HCl was added to A for a 0.8 ml final volume. 73 µl of water and 80 µl of 5N HCl were added to B for a 0.88 ml final volume.

[b]Data shown for two extractions.

Figure 19 PHLD-Catalyzed Hydrolysis of VBI: Double-Labeled, Varying Time. A homogeneous population of VBI of ^{3}H N-methyl choline, ^{14}C 1-fatty acyl labeled PC was prepared in 10 mM PIPES, 10 mM NaCl, 0.36 mM $PrCl_3$, 0.2 mM EDTA pH 6.0 according to the standard method, except that the concentration of phospholipid in ethanol was 40 mM. Elution during gel filtration was with an isotonic buffer substituting NaCl or $PrCl_3$. Chromatographed VBI were incubated with approximately 0.1 units/ml of commercial PHLD, and 0.2 ml aliquots were taken at indicated time points and extracted according to the method of Clancy (31). A aliquot of each organic phase was transferred to a scintillation vial, solvent was removed, and levels of ^{3}H and ^{14}C were determined. The curve which initially *decreases* with time is the ratio [^{3}H-PC] / {[^{14}C-PC] + [^{14}C-PA]}, the uncorrected ratio of respective cpm (closed diamonds). The curves which initially *increase* with time are the calculated molar ratios of PA to PC (open squares) and the mol% PA in PC (closed diamonds). See the text for further information.


Hydrolysis of VBI in the Abscence of Ca^{2+}
1.50
1.25
1.00
0.75
0.50
0.25
0.00
50
40
30
20
10
0
0
1
2
3
4
5
Time (hours)
PA in Total Phospholipid (mol%)
cpm ^{3}H/cpm ^{14}C
[PA] / [PC]
PA in Total PL

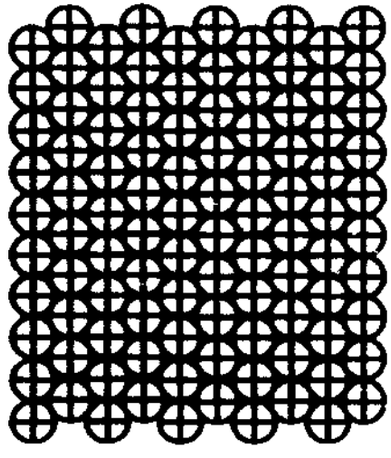
After the reaction, extraction and washing, dried aliquots of the organic phase were dissolved in scintillation fluid and counted. Since all choline is absent from the organic phase, radioactivity is attributed to phospholipid. The ratio of counts in the ^{3}H and ^{14}C is seen as a descending curve, indicating a decrease in the relative ratio of choline to fatty acid in the phospholipids in the sample. The decrease is a consequence of the increasing proportion of PA in the total pool of phospholipid, seen here to approach 30 mol%. This corresponds to a molar ratio of PA to PC of approximately 0.4. A similarly designed fixed-time experiment with varying enzyme concentration gave nearly identical results.

This experiment, viewed synoptically with other VBI experiments, indicates that up to 30 mol% PA can be generated in a preparation of VBI in approximately 90 min. by PHLD-catalyzed hydrolysis in the absence of calcium at pH 6.0. Moreover, these VBI have a trapped shift reagent (Pr^{3+}), and are therefore amenable to further characterization by ^{31}P NMR (see the next section and Conclusions). The vesicles lack divalent cations in the external compartment, and are therefore likely to be stable to redistribution of PA, perhaps up to hours (43).

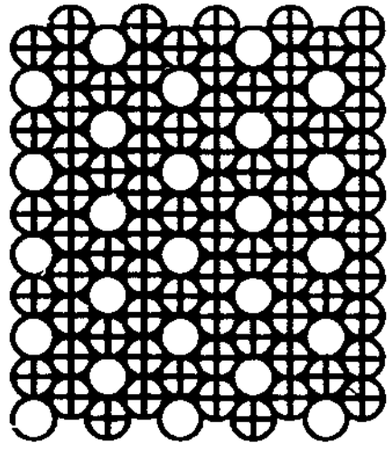
The graphic in Figure 20 represents the increasing surface density of PA in the outer leaflet of a vesicle of ≈90 nm diameter, as observed during the first 30 min. of the time-course in Figure 19. It is assumed that PA formed remains localized in the outer leaflet during this period. After the first quarter hour, the outer leaflet is approximately 25 mol% PA; after an additional quarter hour, the amount increases less dramatically, to about 30 mol%. At its maximum (90 min., not shown), PA composed about 54% of outer leaflet phospholipid.

Such a presentation raises the question of how zwitterionic (PC) and acidic (PA) headgroups pack, as the ratio of PA-to-PC increases in a leaflet. Planar arrays of spherically symmetric regions of neutral and negative charge can adopt two configurations where the criterion of minimum approach

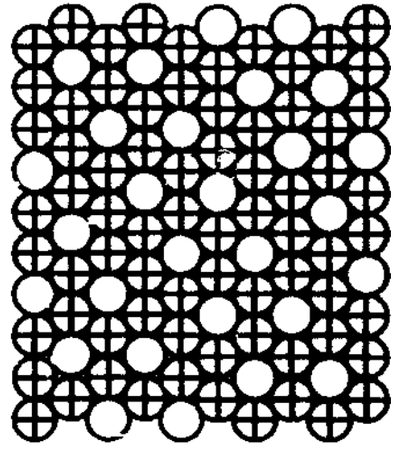
Figure 20 PHLD-Catalyzed Hydrolysis of VBI: Calculated Surface Density of PA in the PC Outer Leaflet. Data from the preceding figure are used to calculate the mol% PA in PC in the outer leaflet, assuming (i) that the ~90 nm vesicles have an outer-to-inner leaflet phospholipid ratio of 1.15 (see Figure 33, Table 12 and accompanying text), and (ii) that no phospholipid flip-flop occurs during the course of the reaction. The data are presented as a 10 x 10 closest-packed matrix of PA (O) in PC (⊠), and suggest an approximate spatial distribution of PA in the PC leaflet as a consequence of the indicated concentration (mol%).



0.00 hours



0.25 hours



0.50 hours

Surface Density of PA in PC in Outer Leaflet: Varying Time.

of the negative charges is met. These configurations correspond to hexagonal and cubic closest packing in crystalline solids. The hexagonal case is used to represent data in Figure 20, and best fits these criteria up to ≈35 mol%. The cubic case with coplanar charge centers placed at the vertices of a square best fits the criteria from 35 - 50 mol%. Above 50 mol% there exists no simple planar arrangement that would separate negatively charged spheres from one another by the requisite distance of two radii.

Modeling the PC and PA headgroups as spherically symmetric regions of neutral and negative charge, repectively, is a simplistic notion, ignoring known features of molecular structure (62, 209, 210) and known intermolecular interactions (211). Yet one might expect that rigorous modeling and theory would agree with the general suggestion that quasi-stable arrangements would change with increasing PA content, perhaps with marked transitions, until a surface density of PA is reached where gross rearrangements which would otherwise be energetically unfavorable are necessary to prevent too close contact between charges. Such an analysis could be useful in explaining the biphasic nature of the time-course of the PHLD-catalyzed hydrolysis of PC in membranes (cf. ref.113), and the sudden gross structural changes that vesicles undergo when concentrations of acidic phospholipid reach threshhold levels.

The use of PHLD to convert PC to PA on the outer leaflet of vesicles thus offers an experimental approach to these questions, as well as to questions of the rate of translocation of asymmetrically distributed PA, and the contribution of an asymmetrically distributed acidic phospholipid to the bulk fluidity of the vesicle membrane. The insight gained from these preliminary studies on the phospholipase D catalyzed hydrolysis of PC in the aqueous/diethyl ether biphase, in dispersions and in vesicles by sonication and injection is summarized in Table 10.

Table 10 Summary of Conditions for the PHLD-Catalyzed Hydrolysis of and Vesicles in the Absence of Calcium.[a]

System[b]	PHLD Preparation	pH	Reaction Duration (hrs.)	mol% PA Produced[c]	Relevant Figures
Vesicles by Injection	affinity	5.6	0.5	5	17
	affinity	5.6	24	33	18
	commercial	6.0	1.5	30	19
	commercial	7.25	24	11	17
Dispersions	commercial	5.5	0.2	10	16
	commercial	6.5	0.2	7	16
Ether Biphase	commercial	7.0	6.0	19	14, 15
	commercial	7.5	6.0	17	14, 15

[a]The substrate in all cases was egg yolk PC. See relevant Figures and Methods for details.
[b]PHLD preparations are from cabbage and are described in Methods; briefly, *affinity* refers to the form described by Clancy (31), purified through a final γ-aminoagarose column, and *commercial* refers to a relatively pure commercial product from Sigma Chemical.
[c]The calculated mol% PA in total vesicle phospholipid: [PA] / [PA] + [PC]. Note that each entry essentially involved a different preparation and concentration of enzyme. Entries should therefore not be compared.

E. Analysis of the Asymmetry of Phospholipids in Vesicles using NMR

1. General

The intended purpose of the NMR experiments reported was to distinguish between the inner and outer leaflets of large vesicles composed of two or more phospholipids. Specifically, it was necessary to resolve the 2-4 characteristic resonances of VBI that might be expected for vesicles that had been incubated with PHLD. The choices available were to study the phosphorus-31 resonance of the phospholipid phosphate of such VBI, or to consider the [choline, N-methyl] and [glycerol, 3-methylene] resonances in their carbon-13 or proton spectra (16).

The ^{31}P nucleus was chosen because of (i) its presence all phospholipid headgroups, (ii) the simplicity of assigning its resonances in spectra of vesicles of more than one phospholipid, (iii) the greater efficiency of its relaxation by paramagnetic ions, over ^{1}H and (iv) its ≈4x greater sensitivity and ≈90x greater natural abundance, over ^{13}C. The choice was made in spite of the larger sample requirement that results from the general low sensitivity of the technique (cf. refs. 16, 84, 67, 176 and standard NMR reference tables). ^{13}C NMR experiments with isotope-enriched phospholipid that supplement the ^{31}P experiments are summarized at the close of this discussion.

As noted in the introduction, ^{31}P NMR can be used to investigate structure and dynamics in vesicles, on time scales defined by the field strength of the instrument employed. In particular, resonance line-shapes contain information on the magnitude and direction of the components of the chemical shift tensor of the absorbing nuclei (cf. refs. 166, 176 here and following). In biological and model membranes, the motion of the nucleus being observed is often anisotropic; corresponding resonance lines are therefore often asymmetric. On the other hand, unilamellar PC

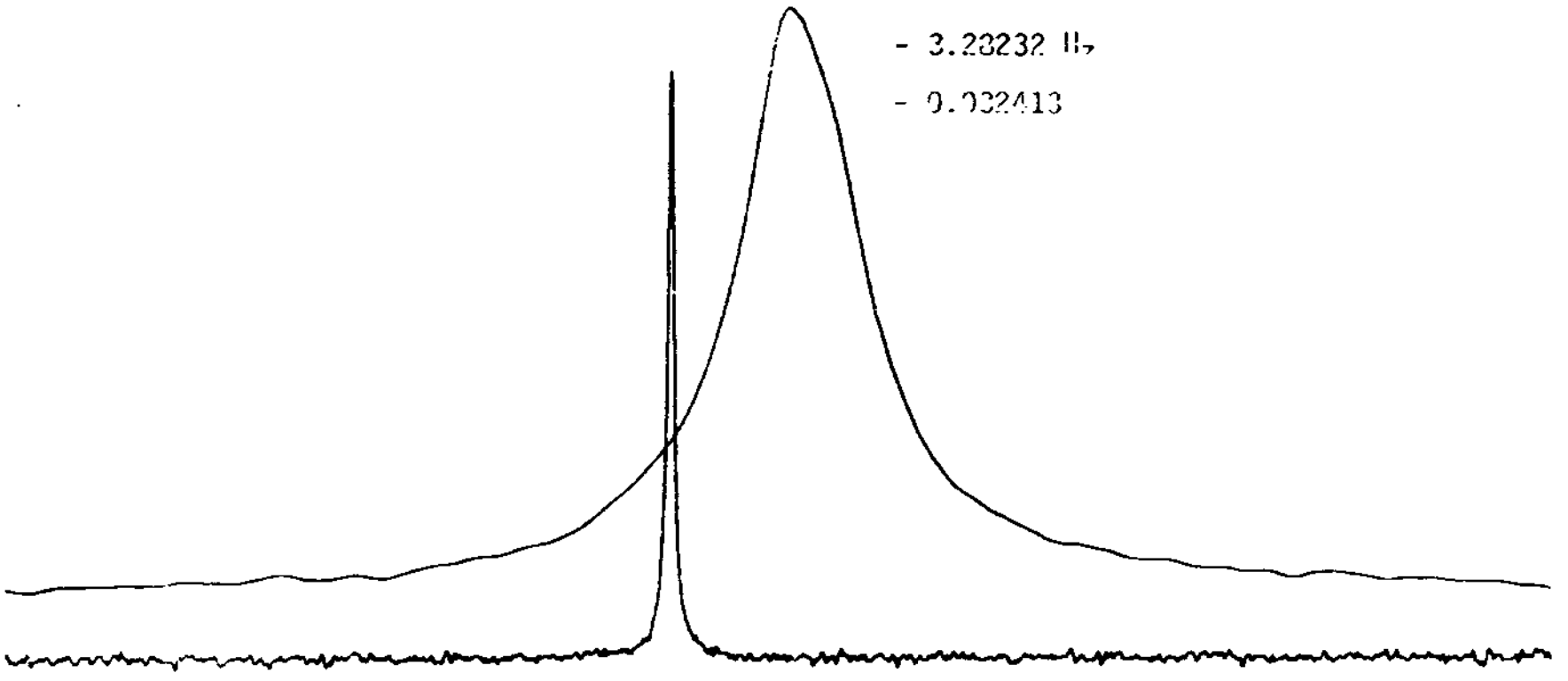
vesicles of the size of VBI or VBS represent an essentially isotropic (motionally-averaged) microenvironment on the NMR timescale; the consequence is a single symmetric resonance (211). Any observed asymmetry of lineshape would suggest a superimposition of two or more lines, reflecting differences in the physical microenvironment of the headgroup phosphorus atom.

Such an asymmetry of lineshape is evident in the upper trace in Figure 21, a spectrum from an early experiment on VBS of egg yolk PC. The single, broadened resonance is composed of overlapping low field (high intensity) and high field (low intensity) components, a characteristic of spectra of VBS. These resonance components have been shown to correspond to the outer and inner leaflets, respectively, and reflect the different headgroup environments due, in this case, to sterically imposed packing differences (150). In addition to interesting structural implications, this observation suggests that the resonances corresponding to the headgroups of the outer and inner leaflets might be resolved by an asymmetric physical or magnetic perturbation of the membrane. If such a perturbation should yield separate resonance intensities proportional to the number of nuclei giving rise to them, then ratios of phospholipid in the outer and inner leaflets could be determined.

The question of resolving these two superimposed resonances was addressed in early investigations of differences in the phospholipid composition between the outer and inner leaflets of membrane bilayers. A significant development was the introduction of paramagnetic shift reagents by L.D. Bergelson, L.I. Barsukov and co-workers (25; reviewed in ref. 16) as tools to distinguish the outer and inner leaflet resonances (hereafter OLR and ILR). The local magnetic fields of such ions or complexes perturb the magnetic environments of nearby nuclei resulting in broadening or shifting of corresponding resonances in the NMR experiment.

Hutton *et al.* (84) present a thorough evaluation of the use of line-shifting reagents for distinguishing OLR and ILR, and conclude that ^{31}P NMR experiments using paramagnetic

Figure 21 High Resolution Phosphorus-31 Fourier Transform Nuclear Magnetic Resonance (^{31}P NMR) Spectrum of VBS above the T_c. The spectrum shown is of an homogeneous population of VBS of egg yolk PC prepared according to the standard method; it is continuous wave (CW) proton decoupled. The phospholipid concentration is approximately 22.5 mM in Na-PIPES. Instrumental parameters were those described for a 25 μsec pulse-width experiments in Methods. 100 acquisitions were averaged. Signal reflects 10 Hz line-broadening. Upper and lower trace-widths are 0.5 and 10 KHz, respectively.


- 3.28232 Hz
- 0.032413

reagents give accurate ratios if several conditions are met. These include (i) sufficient reagent to completely distinguish resonances of interest, (ii) fast exchange of free and bound metal ion, (iii) independence of individual binding events, (iv) an impenetrable and inert bilayer with respect to the reagent, (v) equal and quantitative contribution of all phospholipids to the resonance and (vi) vesicles with homogeneity and integrity unaffected by presence of the reagent. Of these, all are well established for the use of lanthanide cations with phospholipid bilayers and may therefore be taken for granted (ref. 84, and arguments and references therein), with the exception of sufficient reagent, equal and quantitive contribution, and vesicle homogeneity.

The condition of vesicle homogeneity needs to be evaluated for each vesicle preparation; Hutton *et al.* (84) note that "inhomogeneous vesicle preparations will give outside/inside ratios that are weighted averages of the ratios for... individual sizes [of vesicle]." This condition was addressed above in sections on the homogeneity of VBI and VBS. The conditions of equal and quantitative contribution of all nuclei and of sufficient reagent are also discussed by Hutton *et al.* Guaranteeing quantitative contribution requires evaulation of the contribution of nuclear Overhauser effects (NOE) to the intensity of individual resonance lines. Sufficient reagent must be demonstrated empirically for each vesicle preparation and paramagnetic reagent. Experiments were therefore performed to determine conditions necessary to meet these two conditions.

2. Eliminating Phosphorus-31{Proton} Nuclear Overhauser Effects

The nuclear Overhauser effect, or NOE, refers to a change in the magnitude of an observed resonance signal when another spin system that is saturated relaxes by a dipolar transfer of magnetization (also termed polarization) to the nuclei being observed (ref. 130, here and following). The transfer has a strong spatial dependence: the intensity of the observed NOE varies inversely with the sixth power of the internuclear distance. In some experiments where protons are

broadband decoupled to remove the spin-spin splitting of heteroatom resonances, a NOE enhancement of resonances from spins near in space to the saturated protons is often the result (130, 58). The theoretical limit of such a NOE enhancement is defined by the sum of one plus the ratio of the gyromagnetic constants of the saturated and observed spin systems (24).

Yeagle and co-workers (211, 212) have shown that a $^{31}P\{^{1}H\}$ nuclear Overhauser effect arising from acidic, methylene and N-methyl protons of phosphorus oxyacids and their choline and other esters is a general feature of their broad-band decoupled ^{31}P NMR spectra. The theoretical limit of the NOE enhancement (NOEE) of the phosphorus signal in these experiments was noted to be 124%. [$NOE_{max} = 1 + (\gamma_H / \gamma_P) = 3.24$] Reported NOEE values for small molecules in these classes were on the order of 30% for oxyacids, 10-70% for monoesters and 60% for diesters.

As NOEE of up to 40% have been observed in ^{31}P spectra of PC vesicle systems, it was necessary to demonstrate that the contribution of NOE to the ^{31}P line intensity was eliminated before using intensities to determine OLR/ILR ratios (24, 211, 213). This can be accomplished by providing the saturated system with a more effective relaxation pathway, such as by addition of a paramagnetic reagent. Alternatively, it can be accomplished instrumentally by modulating or "gating" the decoupler power during the normal observe sequence. The method proposed to distinguish OLR from ILR in this study requires that the paramagnetic species (here Pr^{3+}) be in contact with only one of the two leaflets of the membrane. Elimination of the NOEE of both OLR and ILR would require exposure of contributing ^{1}H spin systems on both sides of the membrane to the paramagnetic species. A paramagnetic reagent, then, could not serve both of these purposes.

The use of gated proton decoupling (58, 211) was therefore chosen as a means to eliminate NOEE. Specifically, the proton decoupler power was gated on immediately before the excitation pulse was

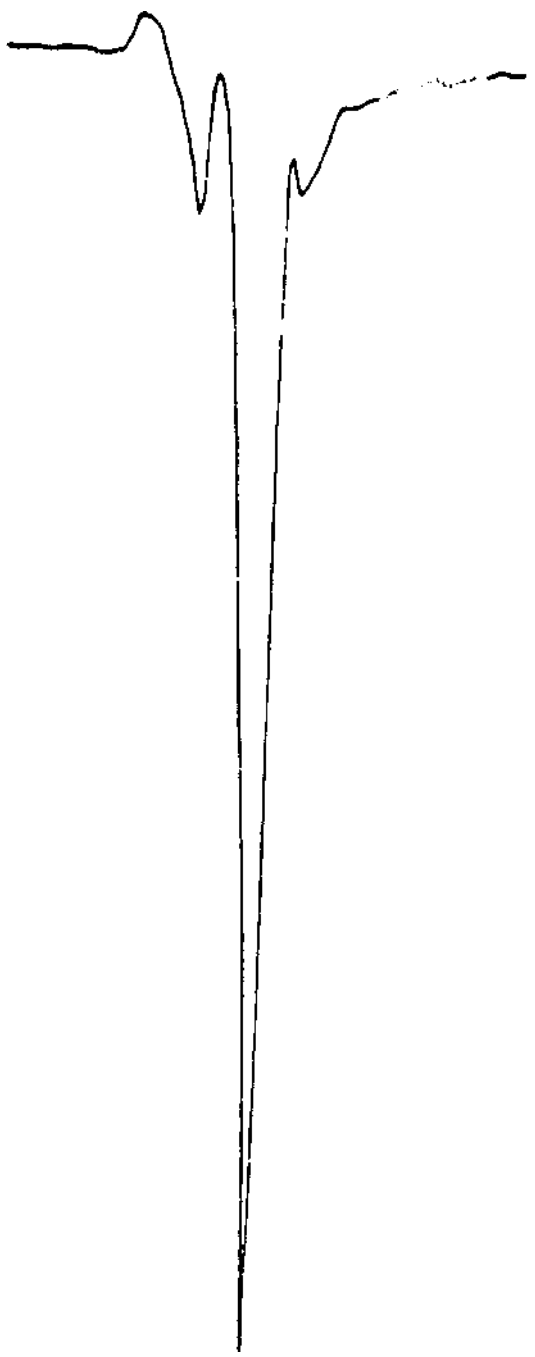
given and gated off immediately after the acquisition of the free induction decay using a Waltz 16 or similar system. The expected result is a proton-decoupled spectrum with NOE eliminated (58). Other instrumental parameters were adjusted to be compatible with this purpose. Long inter-pulse delays were initially used to ensure full relaxation of ^{31}P spins. Generally, chosen delays were on the order of 5 times the known or estimated time constant for the predominant mode of relaxation, here, for spin-lattice or T_1 relaxation. Estimates were based on reported T_1 values for similar systems (196, 211-213). Actual post-acquisition delays used for each experiment in this study are reported in Methods and in the legends to each figure.

The number of acquisitions, NA, was initially estimated based on knowledge of the phospholipid concentration and experience with the sensitivity and natural abundance of phosphorus-31. Data from an experiment of approximately 30 min. per milligram of elemental phosphorus was evaluated (J. Hoots, personal communication). The NA was then refined for each sample such that the ratio of signal to noise was on the order of 50-100. Resonance intensities (integrals) were evaluated by the procedures described in Methods. The percent NOEs reported are based on intensities that were the consensus of data from all procedures. Compared spectra were plotted with identical scaling factors (AK in the Nicolet 1180 software). Spectra for model compounds (Figures 22-31) and for VBS of synthetic and natural PC (Figures 27-28) are presented here. Calculated NOEE values from these spectra and from a spectrum of VBI of natural PC are tabulated.

Spectra of a 1% solution of phosphorous acid trimethyl ester (trimethoxyphosphite) acquired at field strength of 100 MHz[1] are shown in Figure 22. A value for the spin-lattice relaxation time

[1]In the following paragraphs it will be necessary to compare results obtained at a field strengths of ≈2.4 and ≈5.9 Tesla. Standard practice is to refer to field strength by the proton resonance frequency at that field strength. Hence, a field strength of ≈2.4 T is also referred to as a field strength of 100 MHz, where the phosphorus observe frequency is ≈40.5 MHz. A field strength of ≈5.9 T corresponds to a proton resonance frequency of 250 MHz,

Figure 22 Measurement of Nuclear Overhouser Effect Enhancements (NOEE) in Phosphorus-31 NMR Spectra: Phosphorous Acid, Trimethyl Ester. Spectra shown are of a 1% (v/v) solution in $CDCl_3$ with continuous wave (CW) or gated (G) proton decoupling. Spectra were acquired and processed on the Varian XL100 described in Methods. Signals reflect 2 Hz line-broadening. Trace-widths are approximately 250 Hz.



(T_1) of this compound was not available at the time of this measurement but was estimated to be less than 2 seconds. A recycle time of 10 seconds was chosen for this measurement. Under these conditions, the 10% NOEE observed is equivalent to the reported value of 15% (212) given the suggested accuracy of the method (5-10%; see refs. 17, 211).

Spectra of a 25 mM solution of the free acid of glycerophosphate acquired at a frequency of 250 MHz are shown in Figure 23. A post-acqisition delay of 185 seconds was chosen based on the reported minimum T_1 value of 37 seconds reported by Yeagle, *et al.* (211). Under these conditions, the observed 35% NOEE was approximately 60% of the value reported for measurements performed at a 100 MHz field strength. This observation is consistent with the report that the $^{31}P\{^1H\}$nuclear Overhauser effect is independent of field strength in the range of 60-100 MHz, but is inversely proportional to field strength in the range of 100-270 MHz (208, 209). The presence of the minor resonances apparent in these spectra might be explained by the differences in microenvironment due to preferred rotational conformations (e.g., about the C2-C3 carbon-carbon bond), or by the presence of a second stereoisomer at the C-2 position of glycerol. No work was undertaken to address this question.

The next set of experiments were performed to determine the effect of shorter recycle times on the observed NOEE. All experiments were performed at the higher field strength of 250 MHz, where theoretical descriptions of the nuclear Overhauser effect (130, 208) suggest an attenuation of observed $^{31}P\{^1H\}$ NOEE. An attempt was therefore made to acquire data suitable for quantitation in shorter experiments based on shorter recycle times. Shown in Figure 24 are spectra of a 60 mM solution of the sodium salt of phosphorylethanolamine at pH 3.1 acquired with full and gated

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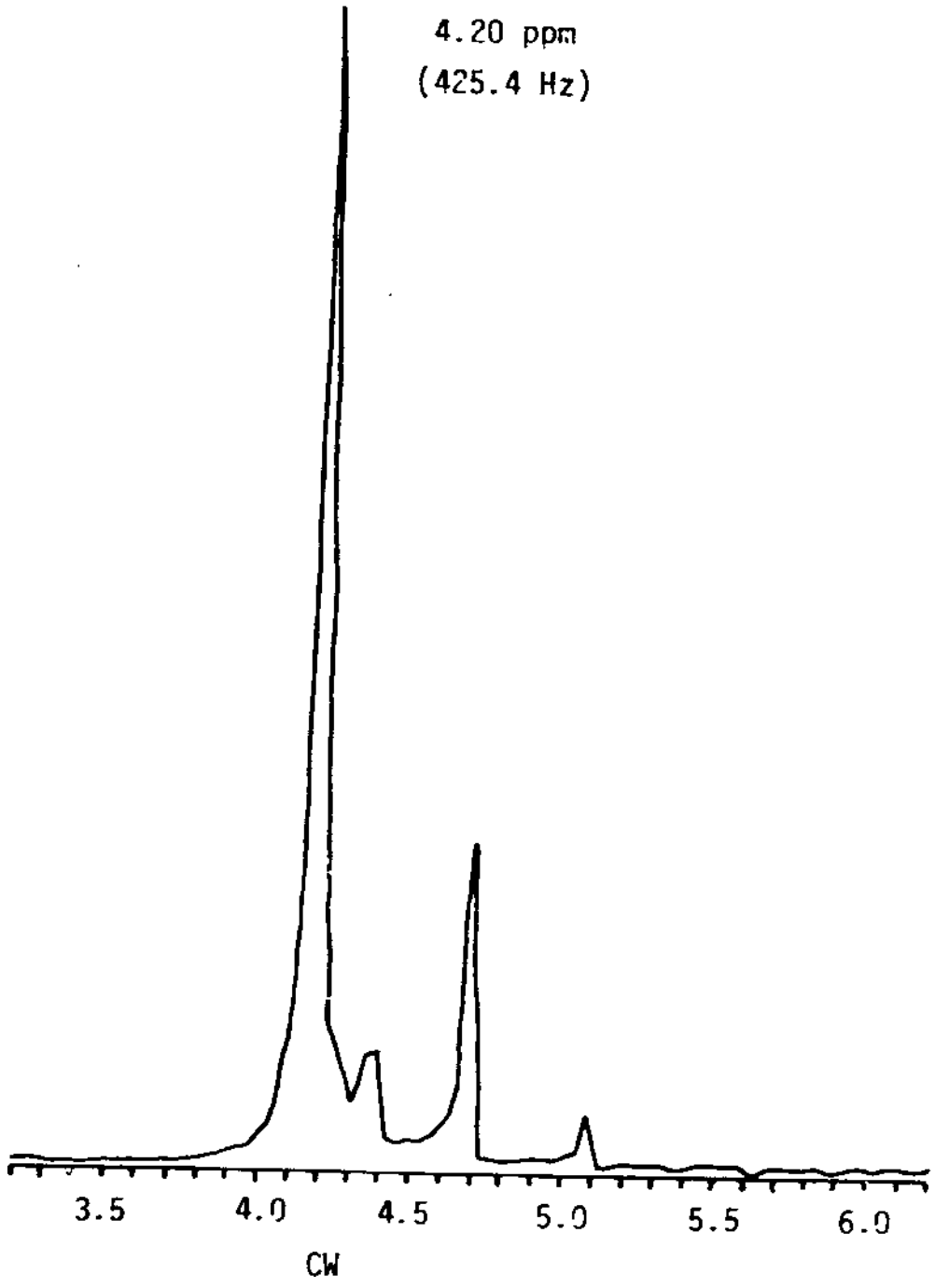
where the phosphorus observe frequency is ~101.3 MHz. For the sake of clarity and simplicity, all comparisons of measurements will list field strength in terms of the proton resonance frequency, i.e. either 100 or 250 MHz. Exact ^{31}P observe and ^1H decouple frequencies are provided in Methods.

Figure 23 Measurement of NOEE in Phosphorus-31 NMR Spectra: Glycerophosphate. Spectra shown are of a 25 mM solution prepared as described in Methods, with continuous (CW) or gated (G) proton decoupling. Instrumental parameters were: 2 KHz spectral width, 1 K data points in the frequency domain, a 42 μsec (≈90°) pulse-width, 250 μsec pre-aquisition delay, 256 msec aquisition time and 105 sec post-acquisition delay . The receiver was gated for 10 μsec after pulse completion. 50 acquisitions were averaged. Signals reflect 1 Hz line-broadening. Trace widths are approximately 3 KHz (30 ppm).


4.20 ppm
(425.4 Hz)
3.5
4.0
4.5
5.0
5.5
6.0
CW

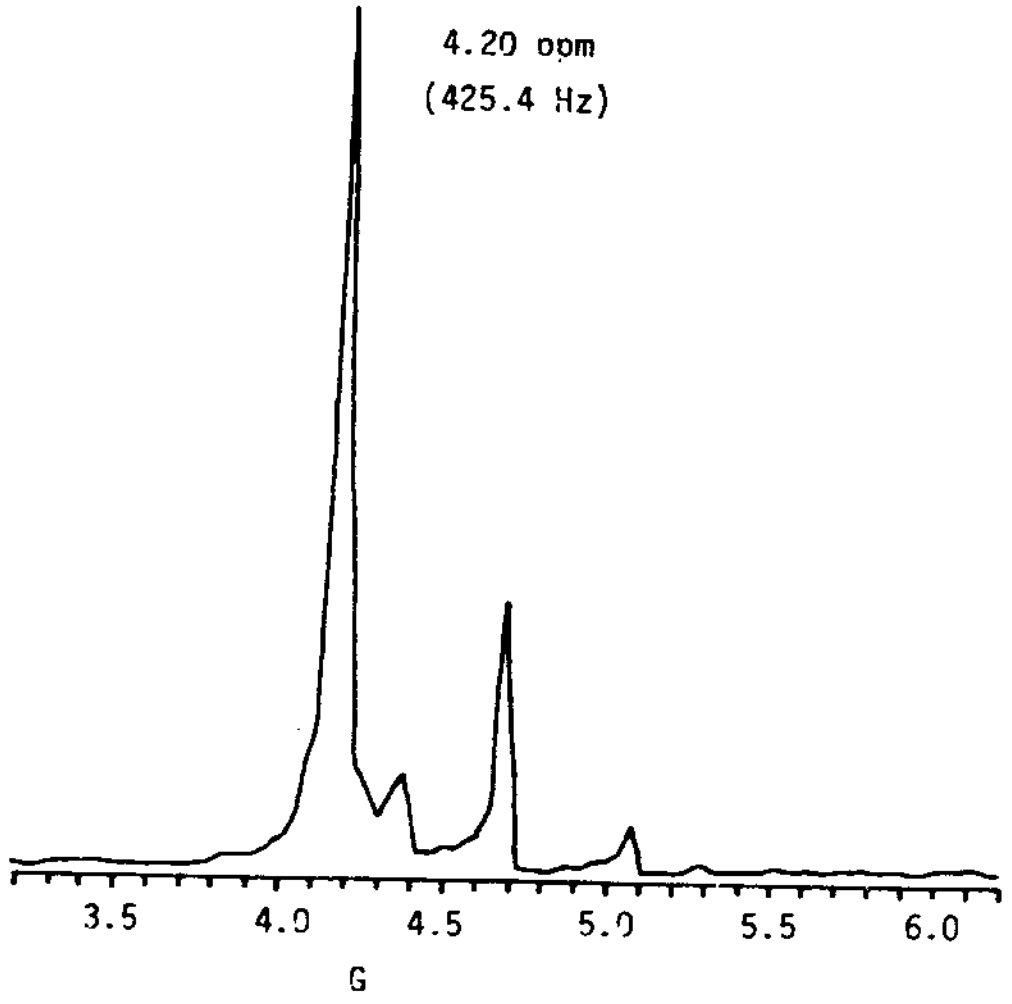
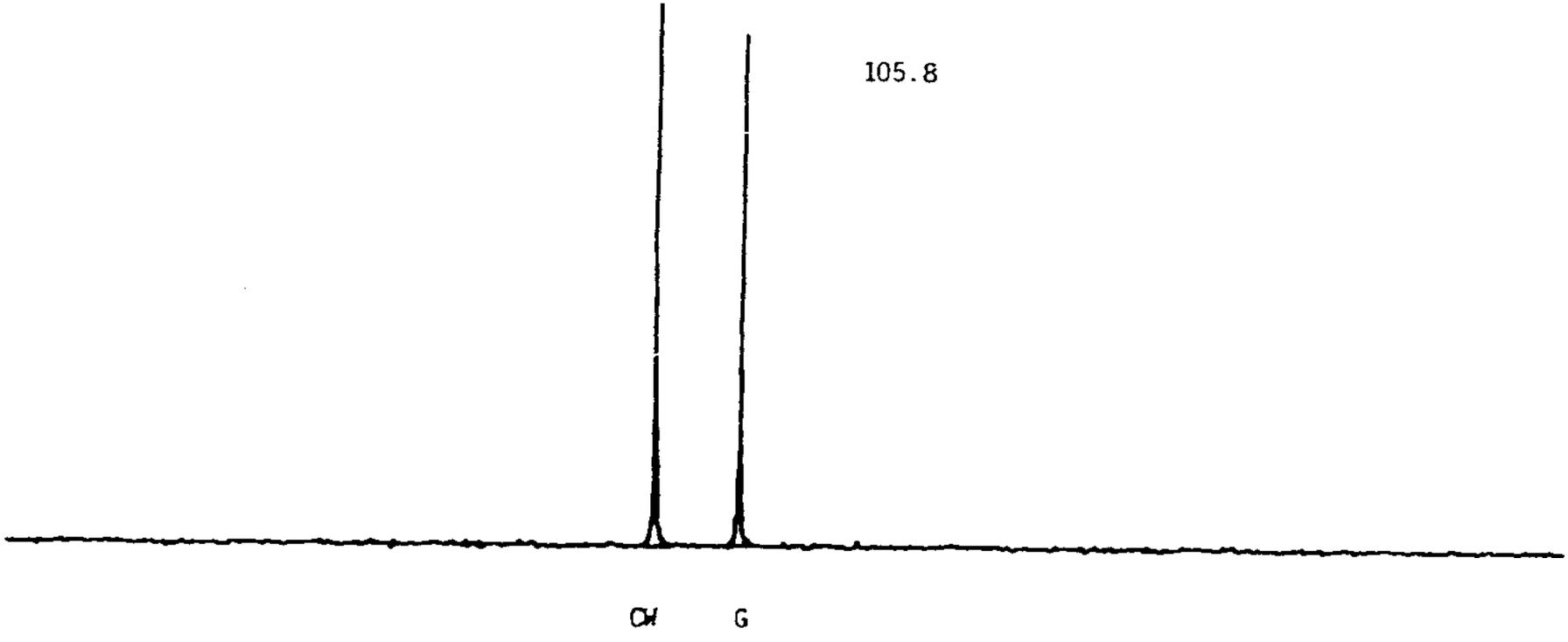

4.20 ppm
(425.4 Hz)
3.5
4.0
4.5
5.0
5.5
6.0
G

Figure 24 Measurement of NOEF in Phosphorus-31 NMR Spectra: Phosphorylethanolamine at pH 3.1. Spectra shown are of a 60 mM sol a of phosphorylethanolamine prepared as described in Methods. Data was acquired with ontinuous-wave (CW) or gated (G) proton decoupling as noted. Instrur al parameters were those described in Methods for a 25 μsec pulse-width experiment, except that 16K data points were taken. 50 acquisitions were averaged. Signals reflect 1 Hz line-broadening. Spectra are overlaid; the chemical shift (in Hz, relative to H_3PO_4) for the two is as noted. The trace-width is approximately 10 KHz.


105.8
CH
G

decoupling. The 2 sec. recycle time chosen was slightly greater than the value of T_1 estimated for this compound. While there are no published reports available to evaluate the observed NOEE of 25%, it is equivalent to the 30% value reported for phosphorylcholine, within the limits of the method. This result encouraged further experiments using short recycle times with well characterized phospholipid analogs and with vesicle systems.

In experiments with phosphorylethanolamine an apparent pH dependence of the phosphorus resonance intensity was noted (Figures 25). The change in resonance intensity pointed to the more significant change apparent in the expanded presentation of these resonances (Figure 26). The pattern of splitting and the coupling constant ($J_3 \approx 3$ Hz) is consistent with coupling of the phosphorus via s orbital interaction with another spin-1/2 system composed of two identical spins. It is most simply explained by the coupling of the phosphorus to two adjacent methylene protons.

The choice of a decoupler power setting insufficient to fully decouple spectra results in spin-spin splitting of the sort seen in the above figures. In quantitative experiments, it can result in distortion of the resonances needed to determine NOEE values, OLR/ILR ratios, etc. On the other hand, recent discussions on the use of NOE measurements to study intermolecular headgroup interactions have suggested that high decoupler power settings can lead to a broadening of the NOE frequency distribution with consequent distortion of NOEE data (209, 24, 211). The phosphoryl-ethanolamine data and the information from the literature suggested that more careful attention be paid to the decoupler power setting, with the need for a distortionless pulse being balanced by the need for sufficient power for complete decoupling.

Spectra of standard preparations of vesicles prepared by sonication of DMPC and egg yolk PC were then acquired using a recycle time of 0.75 seconds at the 250 MHz field strength. These spectra are shown in Figures 27 and 28. Under these conditions, the measured values of the

Figure 25 Measurement of NOEE in Phosphorus-31 NMR Spectra: Phosphorylethanolamine with Continuous-Wave Proton Decoupling. Spectra shown are of a 60 mM solution of phosphorylethanolamine as in Figure 25, except that pH of the sample was adjusted with DCl or NaOD to the values noted below the spectra. Data was acquired with continuous-wave ^1H decoupling; remaining instrumental parameters were as described in Figure 25. Spectra are overlaid; the chemical shifts (in Hz, relative to H_3PO_4) for the three samples are as noted. The trace-width is approximately 10 KHz.

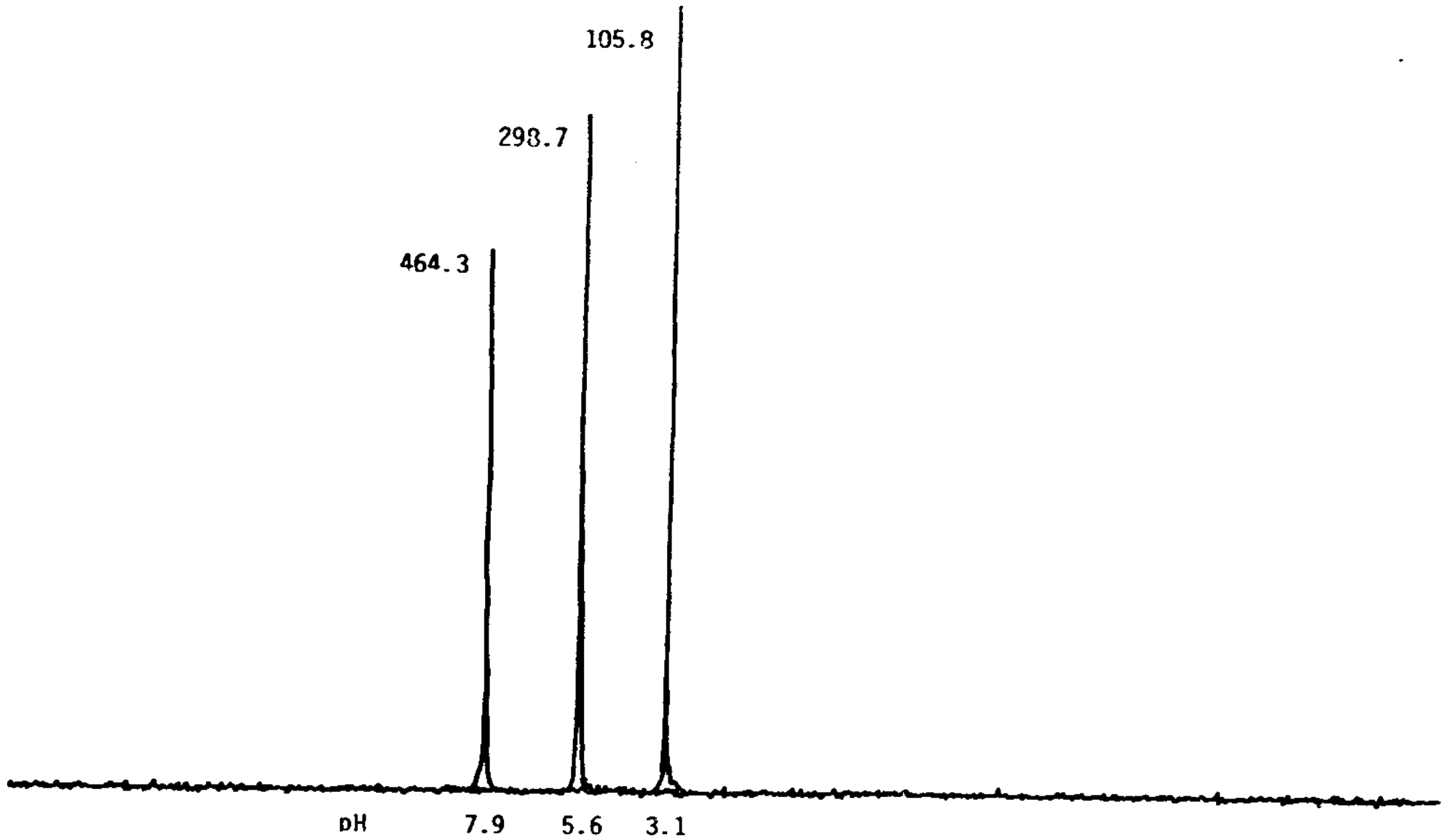
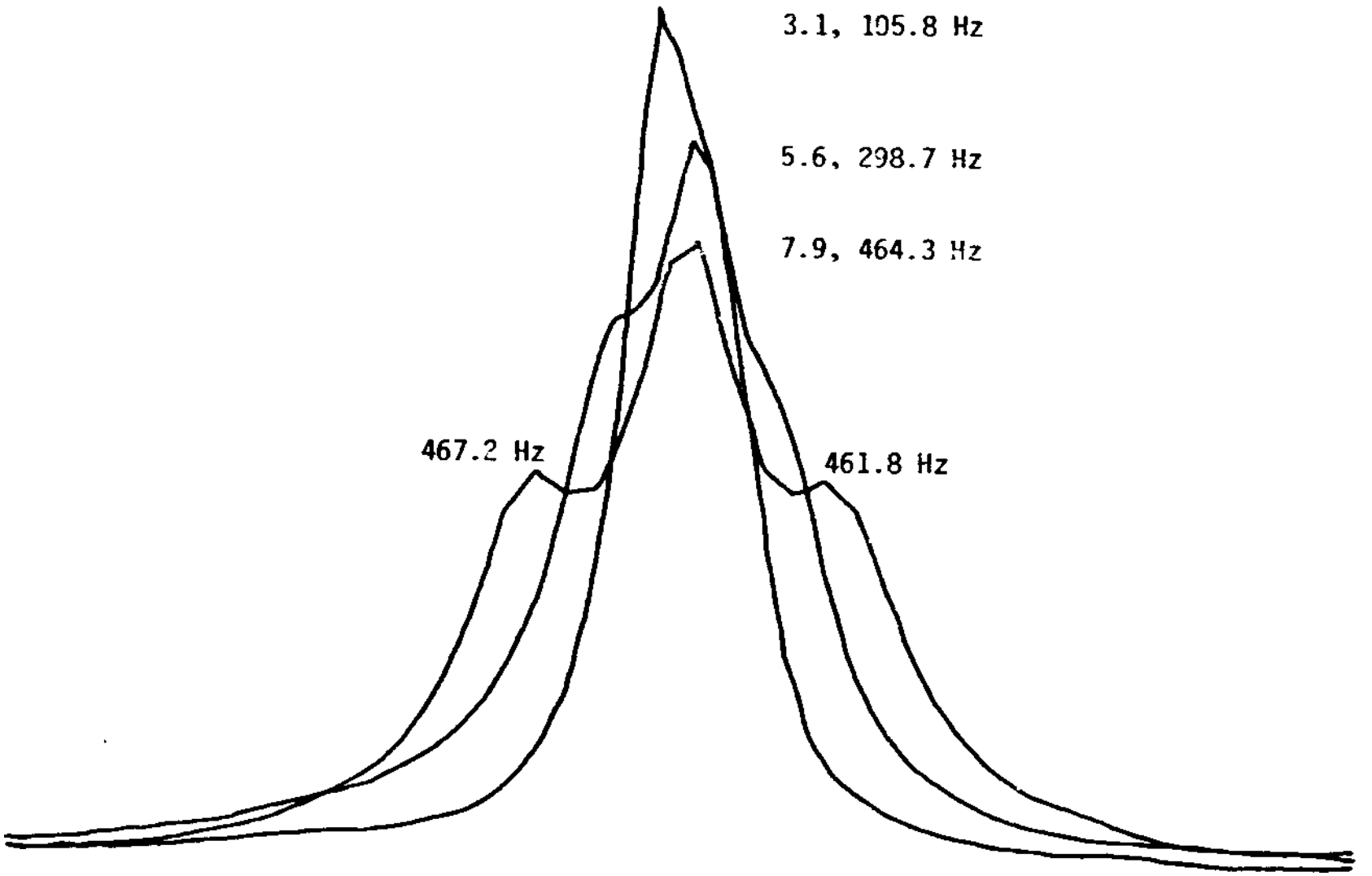

105.8
298.7
464.3
pH
7.9
5.6
3.1

Figure 26 Measurement of NOEE in Phosphorus-31 NMR Spectra: Phosphorylethanolamine with Continuous-Wave Proton Decoupling (Expanded). These spectra are expanded presentations of the data shown in the preceding figure. Spectra are overlaid and centered; pH is noted for each sample (3.1, 5.6 and 7.9), as are the chemical shifts (in Hz, relative to H_3PO_4) of clearly resolved resonances. Trace-widths are approximately 160 Hz. See the preceding two figures for procedural details.


3.1, 105.8 Hz
5.6, 298.7 Hz
7.9, 464.3 Hz
467.2 Hz
461.8 Hz

NOEE were 20% and 10% for DMPC and egg yolk PC vesicles, respectively. The NOEE for the 90 nm vesicles prepared by injection of egg yolk PC (VBI) was approximately equal to that determined for the sonicated vesicle of the same lipid (data not shown).

As noted above and shown in Table 11, there is a decrease of ≈40% in the NOEE value of glycerophosphate at 250 MHz relative to the lower field strength. If this change is a valid reflection of the field-dependent attenuation of the NOE, then other values of NOEE reported here are either exactly comparable or somewhat low relative to published values for vesicle systems. An approximate NOEE for VBS of DMPC of ≈35% at 100 MHz is suggested by extrapolation of the data of Viti and Marinetti (196). An attenuation of ≈40% would suggest an NOEE of 20% at 250 MHz, exactly comparable to the value reported here. Yeagle, *et al.* (211) report the NOEE of VBS of egg yolk PC to be 40% at 25°C from spectra acquired at 100 MHz. Attenuation by ≈40% would indicate an NOEE of 23% at 250 MHz, a value somewhat lower than the 10% reported here.

In any case, the NOEE results were considered close enough to expected values to begin developing the paramagnetic shift method. These further experiments would then provide another means to test for residual NOE in the shorter recycle time experiments, and consequently to test the validity of these data for determining the ratio of outer leaflet resonances to inner leaflet resonances in phospholipid vesicles.

2. Praesodymium (III) in Distinguishing Inner and Outer Leaflet Phosphorus-31 Resonances

In addition to the condition of vesicle homogeneity and of equal and quantitative contribution of nuclei to the observed resonance line, the use of paramagnetic reagents to determine the ratio of

Table 11 Elimination of Nuclear Overhouser Effect Enhancement in Phosphorus-31 Spectra of Model Compounds and Vesicles.

System	Spectrometer Field strength (MHz)	NOEE[a] (%)	T_1 (sec)[b]	Chemical Shift, d (ppm)[c]	Notes, refs.
phosphorous acid trimethyl ester	100	15	nd	nd	this work
	100	10	nd	nd	ref. 212
glycerophosphate	250	35	(37)	+4.20	this work
	100	60	(37)	+4.20	ref. 211
phosphoryl-ethanolamine	250	25	nd	+1.04	this work, pH 3.1
VBS, DMPC	250	20	(1.4)	-0.11	this work
VBS, egg yolk PC	250	10	(1.5)	-0.03	this work
	100	40	1.5	+2.00	ref. 211
VBI, egg yolk PC	250	(10)	(1.5)	-0.15	this work

[a]NOEE is defined as the percent signal enhancement relative to conditions where NOE is eliminated, and is reported to the nearest 10%.

[b]All values of T_1 are from Yeagle *et al.* (211), except for that of VBS, DMPC which is from Viti and Marinetti (196); see text and ref. 211 for discussion.

[c]Chemical shifts (δ) are reported in ppm for sake of comparison, and are relative to 85% H_3PO_4. In this work, referencing was done by substitution. Positive values of δ are in the direction of decreasing field according to IUPAC convention (67).

Figure 27 Measurement of NOEE in Phosphorus-31 NMR Spectra: DMPC Vesicles by Sonication. Spectra shown are of an homogeneous preparation of VBS of DMPC, prepared according to the standard method and acquired with continuous-wave (CW) or gated (G) proton decoupling. Phospholipid concentration is approximately 22.5 mM in Na-TES. Instrumental parameters were those described for a 20 μsec pulse-width experiment. Spectra are overlaid; chemical shift (in Hz, relative to H_3PO_4) is identical for both and is noted. 500 acquisitions were averaged. Signals reflect 10 Hz line-broadening. Trace-widths are approximately 10 KHz.

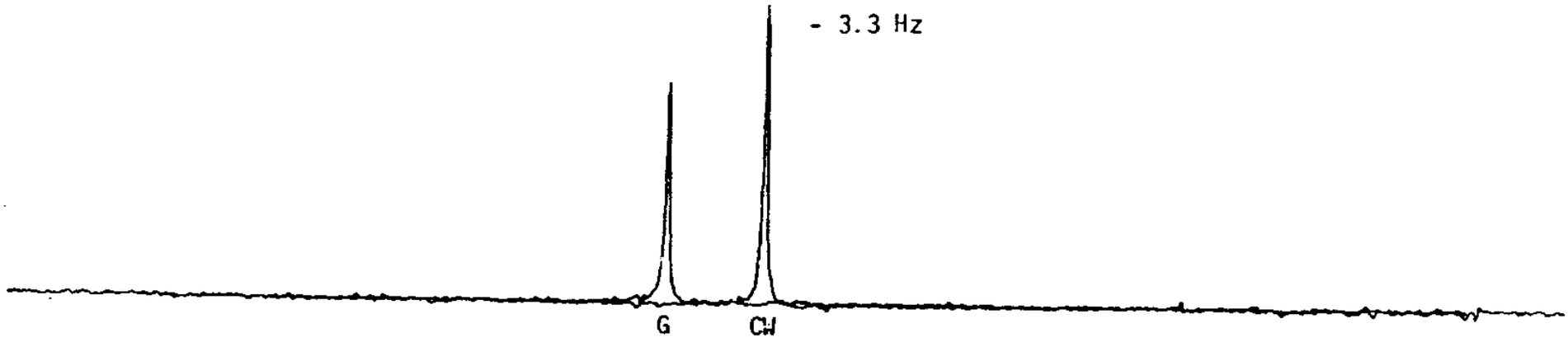
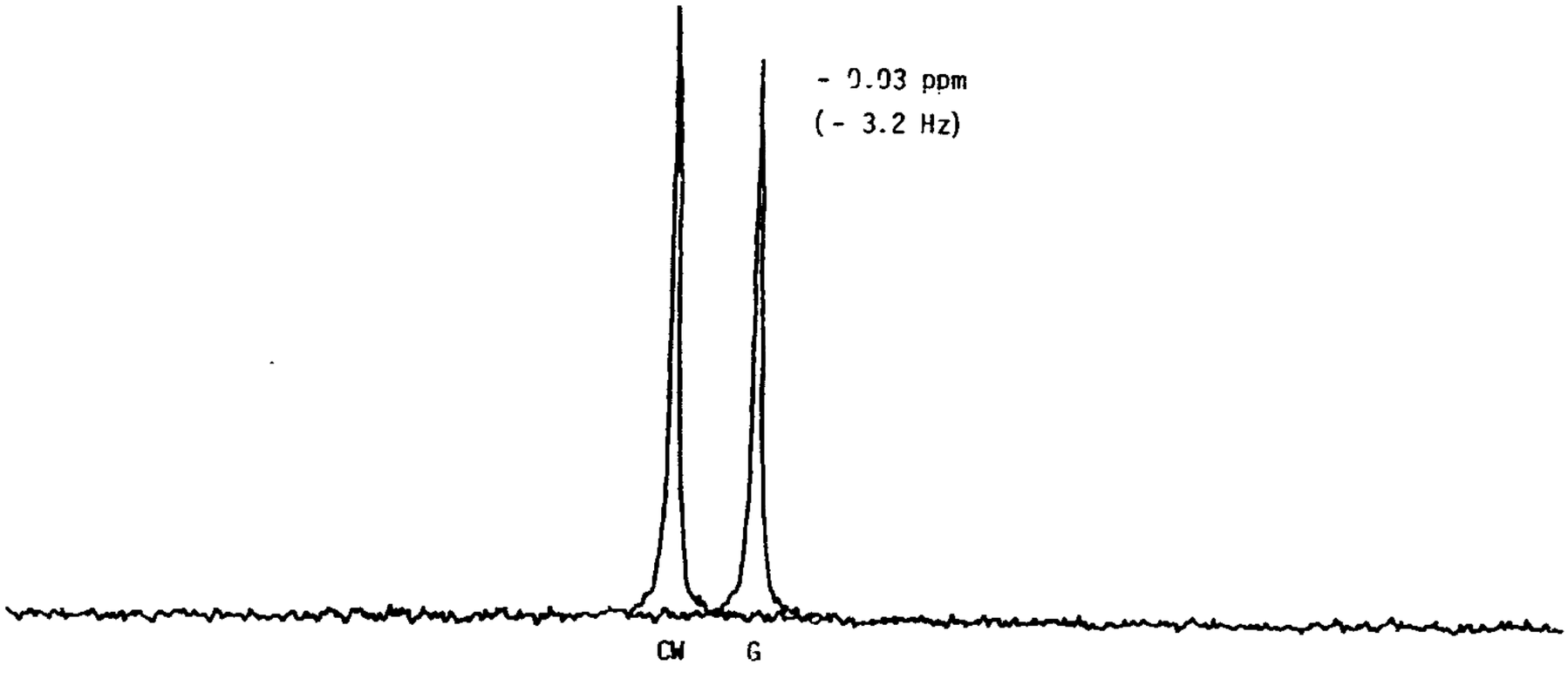

- 3.3 Hz
G
CH

Figure 28 Measurement of NOEE in Phosphorus-31 NMR Spectra: Egg Yolk PC Vesicles by Sonication. The spectra shown are of an homogeneous preparation of VBS of egg yolk PC prepared according to the standard method and acquired with continuous-wave (CW) or gated (G) proton decoupling. The phospholipid concentration is approximately 22.5 mM in Na-PIPES. Instrumental parameters were as described for a 25 μsec pulse-width experiment. Spectra are overlaid; chemical shift (in Hz and ppm, relative to H_3PO_4) is identical for both and is noted. 100 acquisitions were averaged. Signals reflect 10 Hz line-broadening. Trace-widths are approximately 10 KHz.


- 0.03 ppm
(- 3.2 Hz)
CW
G

OLR to ILR requires experimental conditions where added reagent completely distinguishes the resonances of interest, while leaving vesicle homogeneity and integrity unaffected (25, 16). The experimental conditions where OLR and ILR are completely distinguished depend upon the type of the relaxation promoted (distinguished by the different spectral effect of line-broadening or line-shifting) and upon the efficiency ($\Delta\delta/\Delta$[reagent]) and effectivity ($\approx\Delta\delta/\Delta\omega_{1/2}$) of the particular reagent within its class (see ref. 16 and below).

These two classes are reviewed by Bergleson (16), from which the following is condensed. The line-broadening reagents, among them Mn^{2+} and Co^{2+}, most often are high-spin transition metals with long electron spin relaxation times They therefore promote relaxation by spin-spin (dipolar, T_2) mechanisms, resulting in broadened lines. These reagents are thought to effect relaxation by a through-space (field or pseudo-contact) mechanism. Line-shift reagents, principally ferricyanide and the lanthanides cations, have much shorter electron spin relaxation times. These reagents perturb the chemical shielding of the observed nucleus without change in T_2 relaxation, resulting in shifted but unbroadened lines. The line-shift mechanism is thought to be a through-bond communication of electron spin density (Fermi contact).

The choice between these two classes depends upon the nature of the measurement being attempted; relevant here are the following observations. The results of a careful study of the effects of various cations on VBS of egg yolk PC-phosphatidylglycerol has suggested that the most efficient and well-characterized line-broadening agent (Mn^{2+}) acts as a catalyst in the transbilayer redistribution (flipping) of the acidic phospholipid (99). Moreover, line-broadening reagents by nature allow only indirect characterization and quantitation of the broadened resonance. Line-shift reagents were therefore selected for initial attempts to distinguish OLR and ILR.

Of the shift reagents, praesodymium (III) was selected because it is among the most efficient and most effective and is well characterized with respect to its use with VBS (16, 84). The praesodymium salt, $PrCl_3$, was commercially available at apparent high purity. The amorphous, hard, light-green solid was used after removing discolored material and after powdering by mortar and pestle. Solutions generally contained insoluble impurities and were therefore filtered through a 0.22 μm filter before use. Use of the reagent was not hindered by the presence of EDTA or EGTA in buffers of vesicle preparations.

Spectra from a titration experiment in which the Pr^{3+} to phospholipid ratio (PPR) in a standard preparation of sonicated vesicles (VBS) of DMPC is increased by repeated addition of small aliquots of the concentrated stock are shown in Figure 29. The experiment was performed using 0.75 second recycle time as described above, and precautions were taken to ensure that spectra could validly be compared. Chemical shift data indicated that the frequency of the high-field (rightmost) resonance is largely unaffected by the increasing concentration of Pr^{3+} (data not shown). The efficiency of Pr^{3+}, that is, the dependence of the chemical shift of the low-field resonance on Pr^{3+} concentration, is shown by the upper curve in Figure 30. The assignment of the low-field resonance to the the outer leaflet, the biphasic appearance and the approximate inflection point of the titration curve, and the PPR required for complete separation of OLR and ILR (≈0.05) are all completely consistent with published reports (84, 165, 16).

The nature of the process by which Pr^{3+} promotes relaxation has been the focus of considerable discussion. The differences between shift and broadening mechanisms noted above are reviewed by Bergleson (16) and by Hutton *et al.* (84). A change in the slope of the plot of $\Delta\delta$ *vs.* [Pr^{3+}] / [phospholipid] is frequently observed in such titration experiments: note the upper curve in Figure 30, and similar curves in refs. 16, 84 and 165. The change in slope has been suggested to cor-

Figure 29 Effect of Praesodymium Concentration on Phosphorus-31 Resonance Separations and Intensities: Titration Spectra. Shown are spectra of an homogeneous preparation of VBS of DMPC prepared according to the standard method. The phospholipid concentration is approximately 22.5 mM in Na-TES. Traces A through E are of VBS with outer leaflets exposed to increasing [Pr^{3+}] by incremental addition of a concentrated solution; field homogeneity was re-adjusted (shimming was performed) after each addition. The ratio of praesodymium-to-phospholipid in each sample is noted next to the letter designation of each spectrum. Instrumental parameters were as described for 20 μsec pulse-width experiments. 500 acquisitions were averaged. Signals reflect 20 Hz line-broadening. Trace widths are approximately 2 KHz. See Materials and Methods for further details.

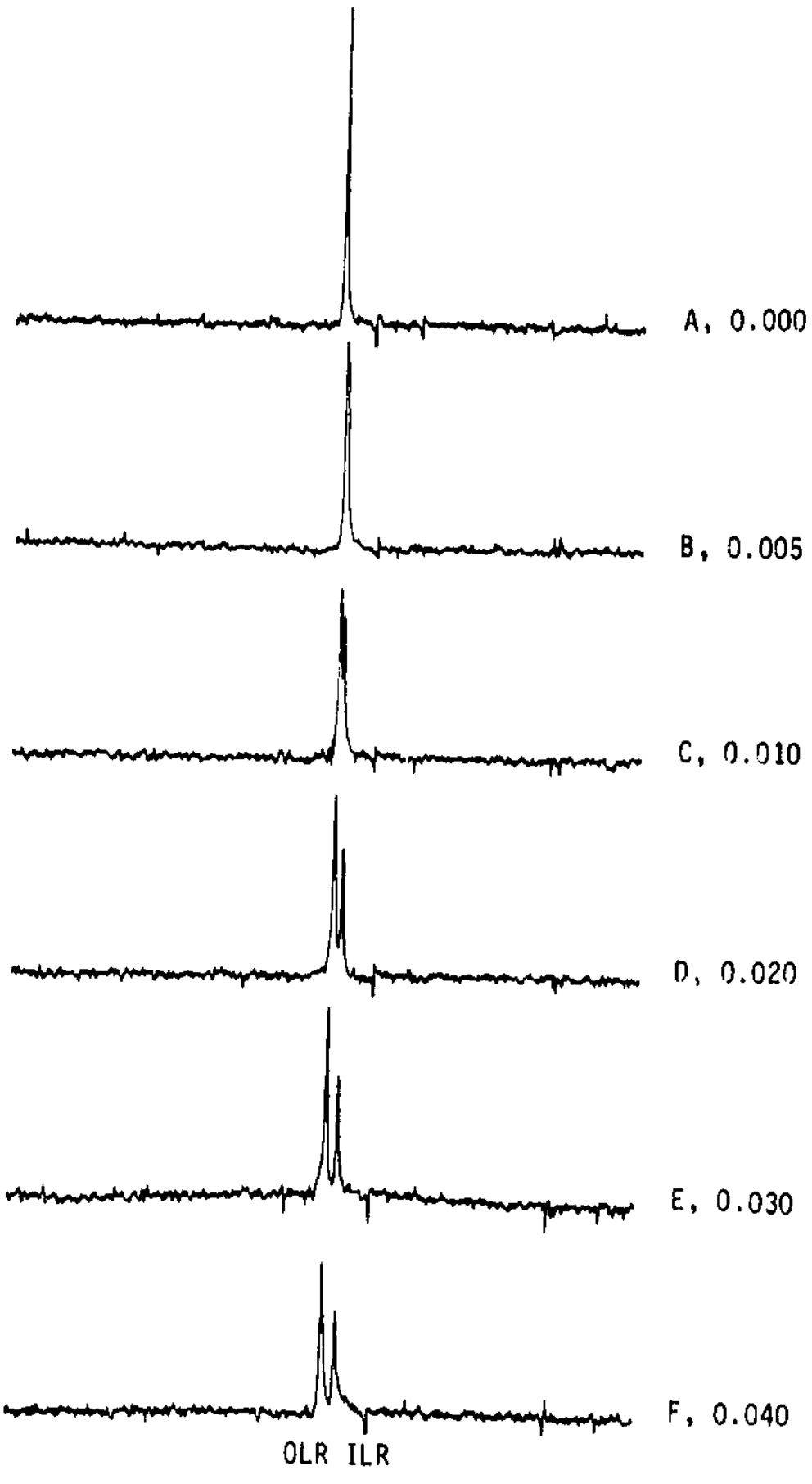
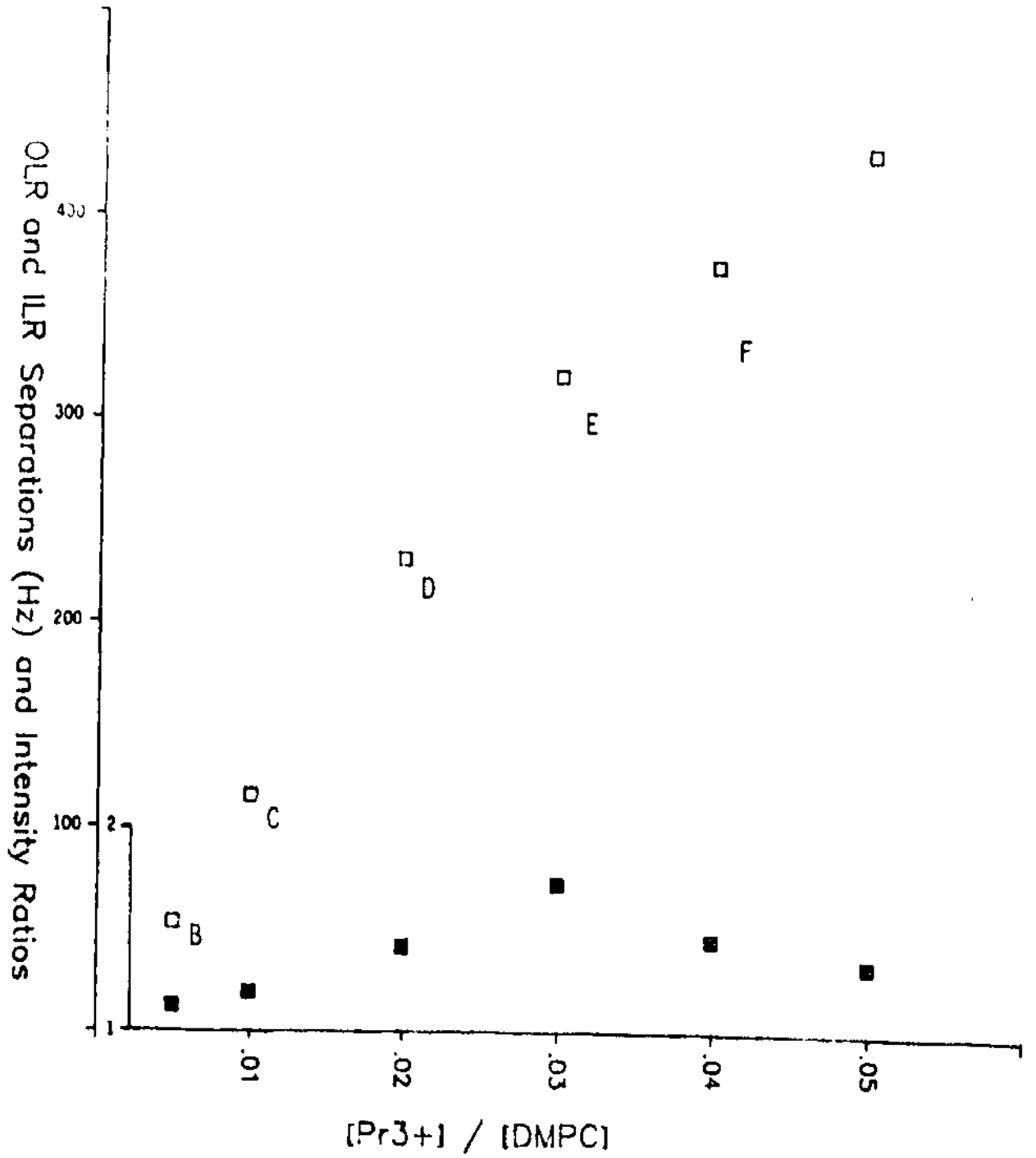

A, 0.000
B, 0.005
C, 0.010
D, 0.020
E, 0.030
F, 0.040
OLR ILR

Figure 30 Effect of Praesodymium Concentration on Phosphorus-31 Resonance Separations and Intensities: Titration Curves. Based on data measured from the spectra in Figure 29, the chemical shift difference ($\Delta\delta$) between outer leaflet (OLR) and inner leaflet resonances (ILR), and the ratio of OLR and ILR peak heights are plotted against the praesodymium-to-phospholipid ratio (squares and crossed-squares, respectively). Letters A through F correspond to the labels of the spectra in Figure 29. See the text for interpretation and discussion.

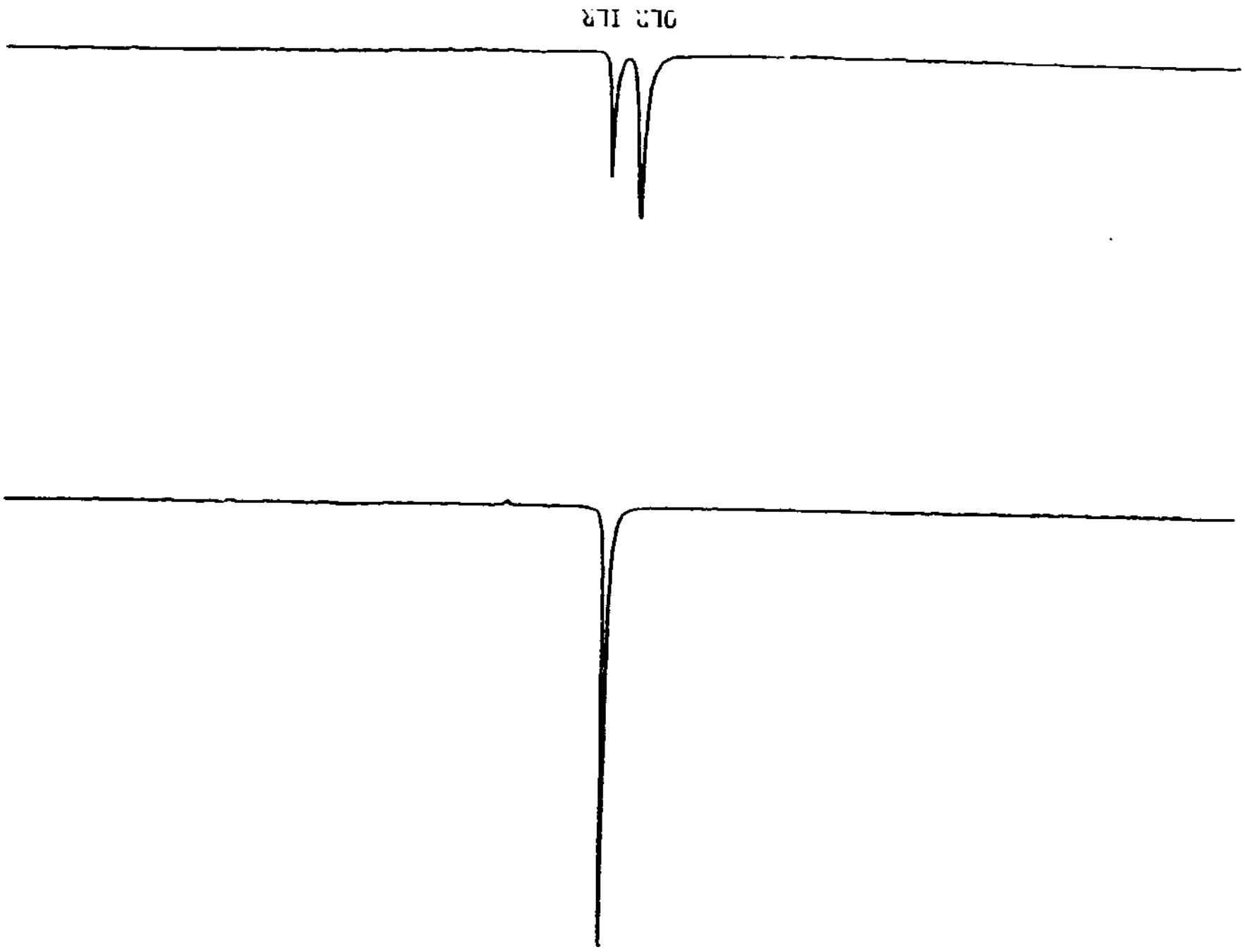

OLR and ILR Separations (Hz) and Intensity Ratios
400
300
200
100
2
1
A
B
C
D
E
F
.01
.02
.03
.04
.05
[Pr3+] / [DMPC]

respond to a change in the nature of the predominant ^{31}P relaxation process at higher Pr^{3+} concentrations, though no experiments have been reported which test this hypothesis.

The lower curve in Figure 30 presents the change in the ratio of peak heights of the OLR to the ILR with increasing Pr^{3+}. In the case that the effectivity of the relaxation was independent of Pr^{3+} concentration, a simple shift of the upfield resonance with increasing Pr^{3+}, and consequently a curve with a positive slope, would be observed. It is apparent that at the approximate praesodymium-to-phospholipid ratio (PPR) where the slope of the efficiency changes, the ratio of the heights of OLR to ILR begins to decrease. This is manifest in the actual spectra by a decrease in the degree to which the added aliquot shifts and an increase in the degree to which the added aliquot broadens the OLR. This apparently novel observation supports the contention that the change in slope of the efficiency curve is a result of a change in the nature of the balance of relaxation processes with increasing shift reagent (165, 84, 16). Specifically, it suggests that at a critical value of the ratio of shift reagent to phospholipid (≈ 0.02 - 0.04), relaxation shifts in predominance from a through-bond Fermi-contact to a through-space spin-spin (T_2) mechanism.

After establishing the praesodymium-to-phospholipid ratio necessary for complete resolution, a number of experiments were performed to demonstrate that (i) the initial results were free from bias due to the asymmetry in ionic strength created by exogenous addition of shift reagent, and (ii) to develop a routine method to produce VBS and VBI in the presence of praesodymium. The result of changing the external buffer of a standard preparation of VBS of DMPC from Na-PIPES to Pr-PIPES with praesodymium-to-phospholipid ratio = 0.050 is shown in Figure 31. Ionic strength of the two buffers was balanced using the equation suggested by Clancy *et al.* (32). The experiment was performed with gated proton decoupling at a power of 2 watts and a recycle time of 0.75 seconds. The OLR and ILR are clearly separable and quantifiable under these conditions.

Figure 31 Effect of Praesodymium Concentration on Phosphorus-31 Resonance Separations and Intensities: Vacuum Dialyzed VBS. Spectra shown are of an homogeneous preparation of VBS of DMPC prepared according to the standard method. The initial phospholipid concentration was approximately 30 mM. The upper trace is of VBS prepared in Na-PIPES; the lower trace is of the VBS after vacuum-dialysis against Pr-PIPES, where the ratio of praesodymium-to-phospholipid = $[Pr^{3+}] / [DMPC] = 0.050$. Instrumental parameters were those described for a 20 μsec pulse-width experiment. Decoupler power was 2 watts. 2000 acquisitions were averaged. Signals reflect 20 Hz line-broadening. Trace-widths are approximately 20 KHz.

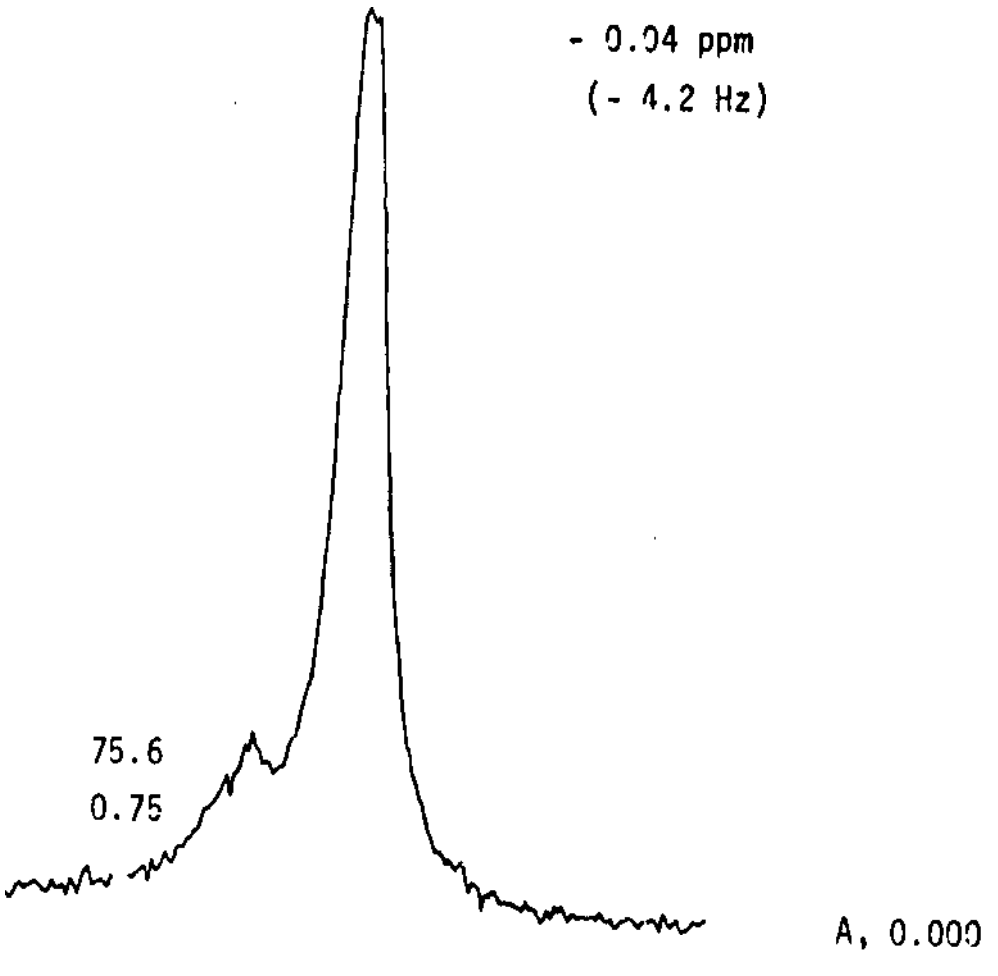

OLR ILR

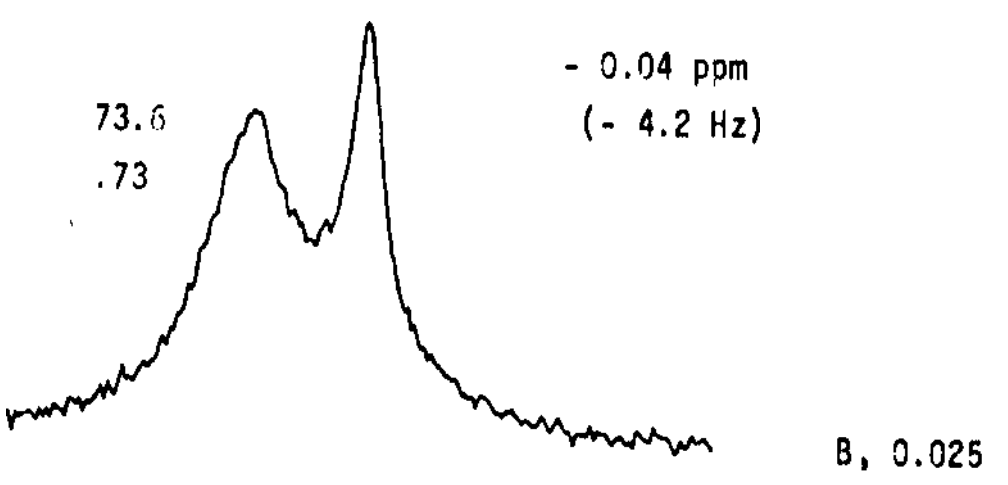
Note that a shift on the order of 30 Hz (0.3 ppm) was observed for the OLR of PC. The difference in chemical shift between PC and PA in VBS composed of these phospholipids is ≈80 Hz (0.8 ppm; cf. refs. 43, 36, 14). One would therefore expect that a shift of the two OLR of PC and PA by the amount seen in the earlier titration experiment would result in resolved resonances for the four predicted microenvironments, apart from the possible specific PA-cation ion interactions. A preliminary fully-decoupled titration experiment was therefore performed; VBS composed of 10 mol% DMPA in DMPC were prepared with only outer leaflet exposed to shift reagent. A praesodymium-to-phospholipid ratio of ≈ 0.05 was used.

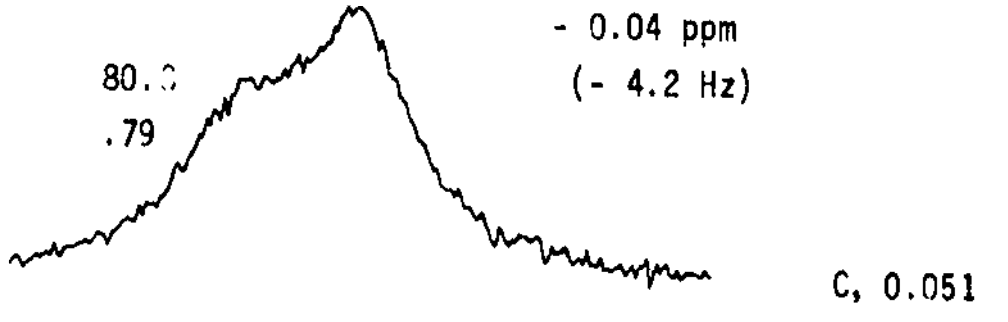
Vesicles produced under these conditions are suggested to approximate VBS of PC in diameter and to have a comparable OLR/ILR when total phospholipid is considered (14). Significantly, vesicles of this type and composition have been shown to be asymmetric with respect to the distribution of PA: PA is found predominantly, although not exclusively, in the inner leaflet (14, 135). Addition of cations (particularly Ca^{2+}) has been shown to perturb the bilayer of such membranes, especially when exposed to the leaflet or leaflets containing PA (26, 36, 43, 69, 74, 86, 101, 125, 133, 137). One might therefore expect that direct exposure of the PA-containing leaflet to Pr^{3+} could result in aggregation or other macroscopic change in vesicle integrity.

The results of the PA/PC-VBS titration experiment are shown in Figure 32. The presence, lineshape and chemical shift of the broad, low-field resonance seen in the top trace is that expected for the PA component in both leaflets (43, 14). As seen in the middle trace of this figure, the addition of Pr^{3+} to praesodymium-to-phospholipid ratio = 0.025 results in a clear shift of intensity from the high field to a low-field resonance, but no clearly discernible third and fourth resonances. The further addition of Pr^{3+} to the ratio of 0.051 shown in the bottom trace results in a relative

Figure 32 Effect of Praesodymium Concentration on Phosphorus-31 Resonance Separations and Intensities: DMPC / DMPA VBS. Spectra shown are of an homogeneous preparation of VBS of DMPC / DMPA (9:1), prepared according to the standard method. The phospholipid concentration is approximately 22.5 mM in Na-TES. Traces A through C are of VBS with the outer leaflets exposed to increasing concentration of praesodymium, by incremental addition of a concentrated solution. The ratio of praesodymium-to-phospholipid in each sample is noted next to the letter designation of each spectrum. Values of chemical shifts appear adjacent to the resonance to which they correspond. The frequency in Hz appears in parentheses; other values are the corresponding shifts in ppm (both relative to H_3PO_4). Instrumental parameters were those described for a 20 μsec pulse-width experiment. 2000 acquisitions were averaged. Signals are unconditioned. Trace widths are approximately 400 Hz.


- 0.04 ppm
(- 4.2 Hz)
75.6
0.75
A, 0.000


- 0.04 ppm
(- 4.2 Hz)
73.6
.73
B, 0.025


- 0.04 ppm
(- 4.2 Hz)
80.3
.79
C, 0.051

broadening of both resonances, without clear evidence of a further shift. No apparent aggregation or precipitation was noted during the course of the experiment. Nevertheless, these spectra suggest that the exposure of outer leaflet PA to Pr^{3+} is responsible for broadening and incomplete shifting of the outer leaflet resonances.

A final ^{31}P experiment was performed to demostrate that the methods developed and published for VBS could also be applied quantitatively to preparations of the 90 nm VBI. The result of changing the external buffer of a standard preparation of VBI of egg yolk PC from Na-PIPES to Pr-PIPES with praesodymium-to-phospholipid ratio = 0.055 is shown in Figure 33. A sharp electronic noise spike of characteristic frequency was edited from this spectrum for the sake of presentation. Again, the OLR and ILR are clearly separable and quantifiable under these conditions. The line widths seen here are broader than those seen for VBS, consistent with the large T_2 values one might expect for large, more slowly tumbling vesicles with longer correlation times. The T_2 relaxation-based broadening of the OLR at the praesodymium-to-phospholipid ratio required to distinguish the two resonances is seen here as it was with VBS.

The unsuccessful attempt to resolve and quantify phospholipid species in PC/PA vesicles exposed to Pr^{3+}, and the successful application of published shift methods to the 90 nm VBI lead to phospholipase D hydrolysis experiments performed on 90 nm vesicles with trapped Pr^{3+}. These successful experiments were performed with two radioisotopic labels, as noted in Figure 19 above. Unfortunately, companion NMR experiments on the product of this type of hydrolysis yet to be performed.

The representative calculated ratios of outer leaflet resonance intensities to inner leaflet resonance intensities for PC vesicles summarized in Table 12 highlight the potential of ^{31}P NMR in

Figure 33 Effect of Praesodymium Concentration on Phosphorus-31 Resonance Separations and Intensities: Vesicles by Injection. Spectra shown are of an homogeneous preparation of VBI of egg yolk PC prepared in Na-PIPES according to the standard method. The outer leaflet was exposed to shift reagent by rapid Sephadex G25 gel filtration chromatography. Following elution with isotonic Pr-PIPES, vesicles were vacuum-dialyzed and concentrated against isotonic Pr-PIPES at a praesodymium-to-phospholipid ratio = $[Pr^{3+}]$ / [egg yolk PC] = 0.055. Phospholipid concentration was approximately 31 mM. Instrumental parameters were those described for a 25 μsec pulse-width experiment. 500 acquisitions were averaged. Signals reflect 10 Hz line-broadening. Trace-widths are approximately 10 KHz. The horizontal bar below the trace reflects a region of the spectrum corrected for software noise; see the text for details.

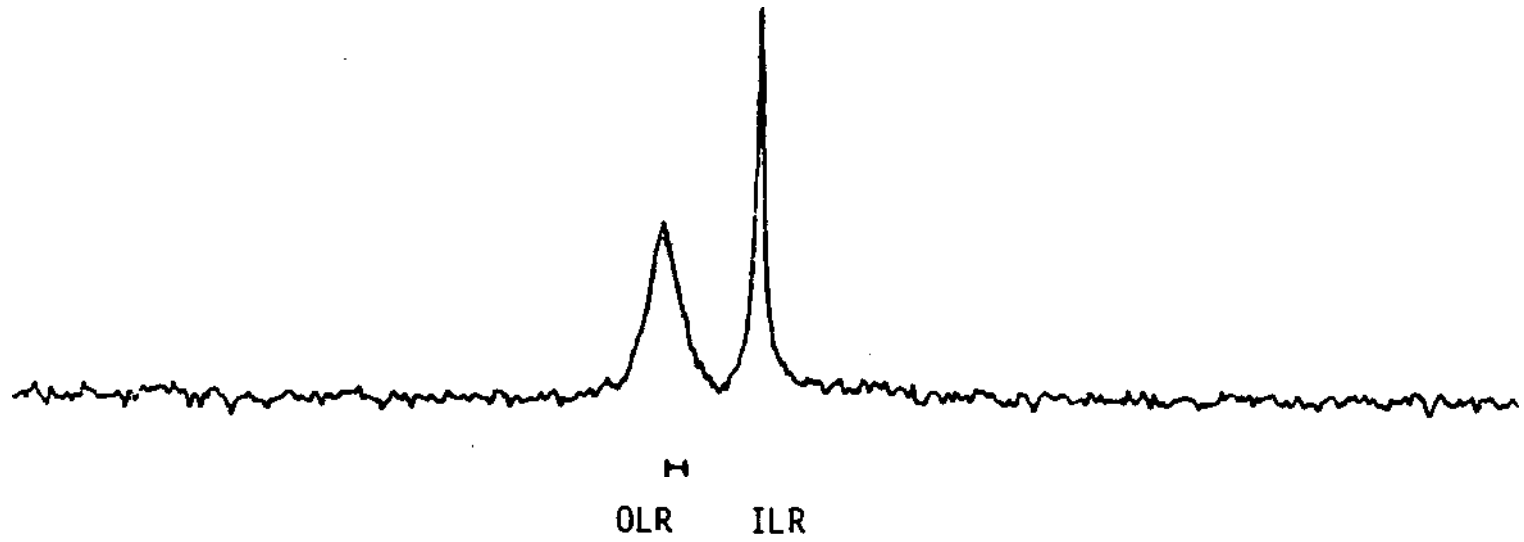

A
H
OLR
ILR

Table 12 Effect of Praesodymium concentration on Phosphorus-31 Resonance Separations Intensities: Ratios of OLR to ILR Intensities.[a]

	Vesicle, phospholipid	OLR / ILR
A.	VBS, DMPC	1.95
B.	VBI, egg PC	1.10

[a]Ratio A is from a spectrum (Fig. 31) acquired with gated ^1H decoupling. Ratio B is from the spectrum in Figure 33 and was acquired with continuous wave ^1H decopling and the ILR is therefore corrected for 10% enhancement due to NOE. Uncorrected ratio 1.0; see text for discussion.

[b]OLR and ILR intensities are the consensus of areas calculated by triangulation and of weights of resonances cut from photocopies of spectra.

discriminating between and quantitating phospholipid species. Here, the experiment to produce the ratio for VBS was performed with a recycle time was 0.75 sec. Significantly, the calculated ratio of 1.95 is within the reported accuracy of repeated, careful determinations (2.00 $\pm$ 0.05, ref. 84), suggesting again that error due to the use of short recycle times was small. The experiment to produce the OLR/ILR of VBI was performed with continuous rather than gated decoupling, also with recycle time of 0.75 sec. The calculated ratio was 1.00, or 1.10 if corrected for the 10% NOEE of the ILR expected in the abscence of gated decoupling. These values are in accord with the ratio of OLR to ILR derived from surface area calculations, which suggest a ratio of 1.15 for 88 nm vesicles of egg yolk PC (see Lichtenberg *et al.*, ref. 116). The value calculated for VBS of egg yolk PC using this method of calculation was 1.94.

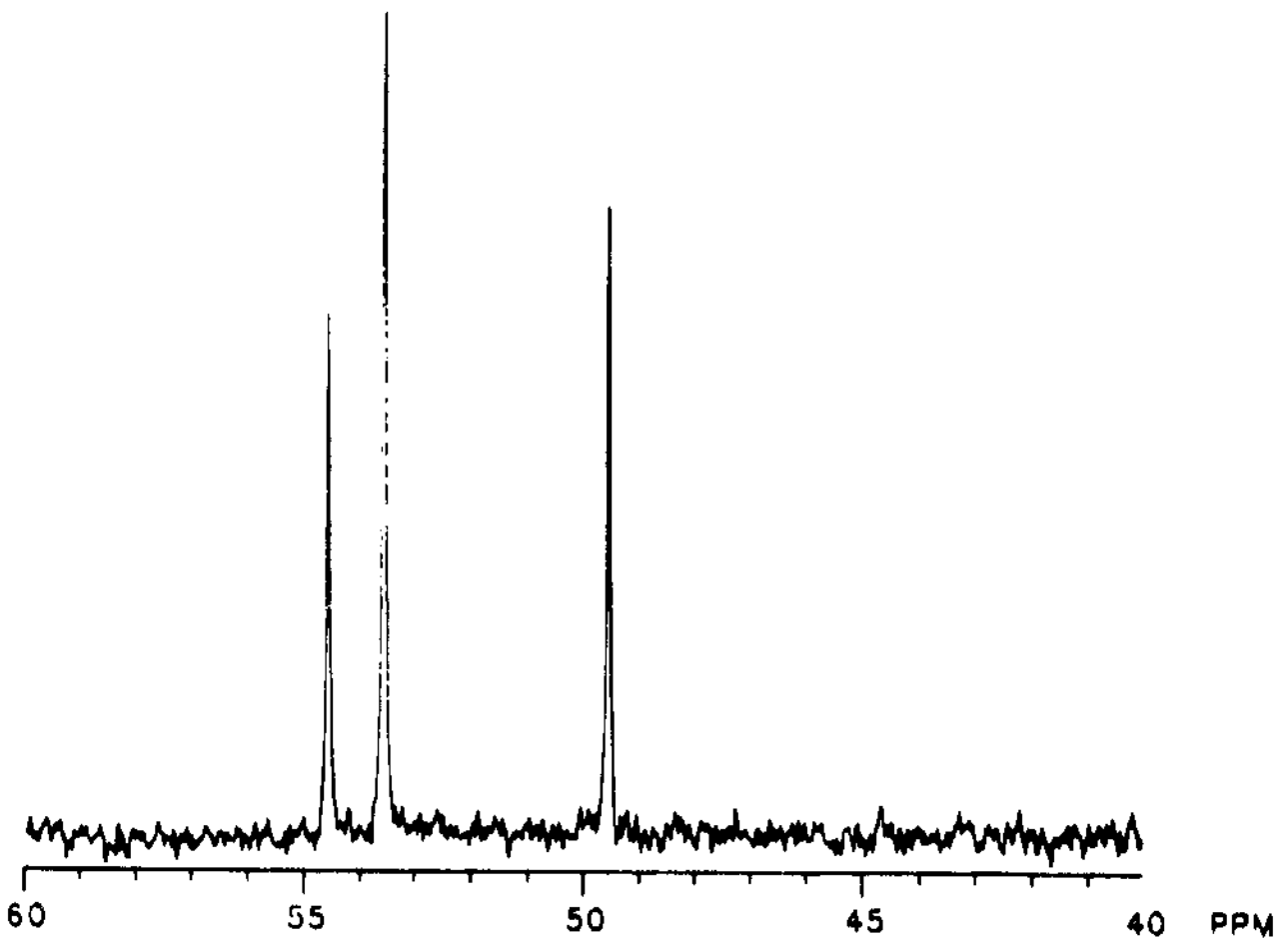
3. Ytterbium (III) in Distinguishing Inner and Outer Leaflet Carbon-13 NMR Resonances

The ^{31}P experiments were supplemented by ^{13}C experiments performed with phospholipid enriched to >95% with ^{13}C in the N-methyl groups of the choline headgroup. Measurement of OLR and ILR intensities by this method have been used to distinguish asymmetries in VBS of PC arising from the presence of acyl chain unsaturation (213). The use of ytterbium (III) as a shift reagent in the capacity suggested here was described by Sears *et al* where a metal-to-phospholipid ratio of 0.06 was observed to be sufficient to completely resolve OLR and ILR (165). The availability of this reagent and of ^{13}C-labeled phosphatidylcholine made it possible to design an experiment to evaluate ^{13}C NMR spectra as a means of rapidly characterizing vesicles.

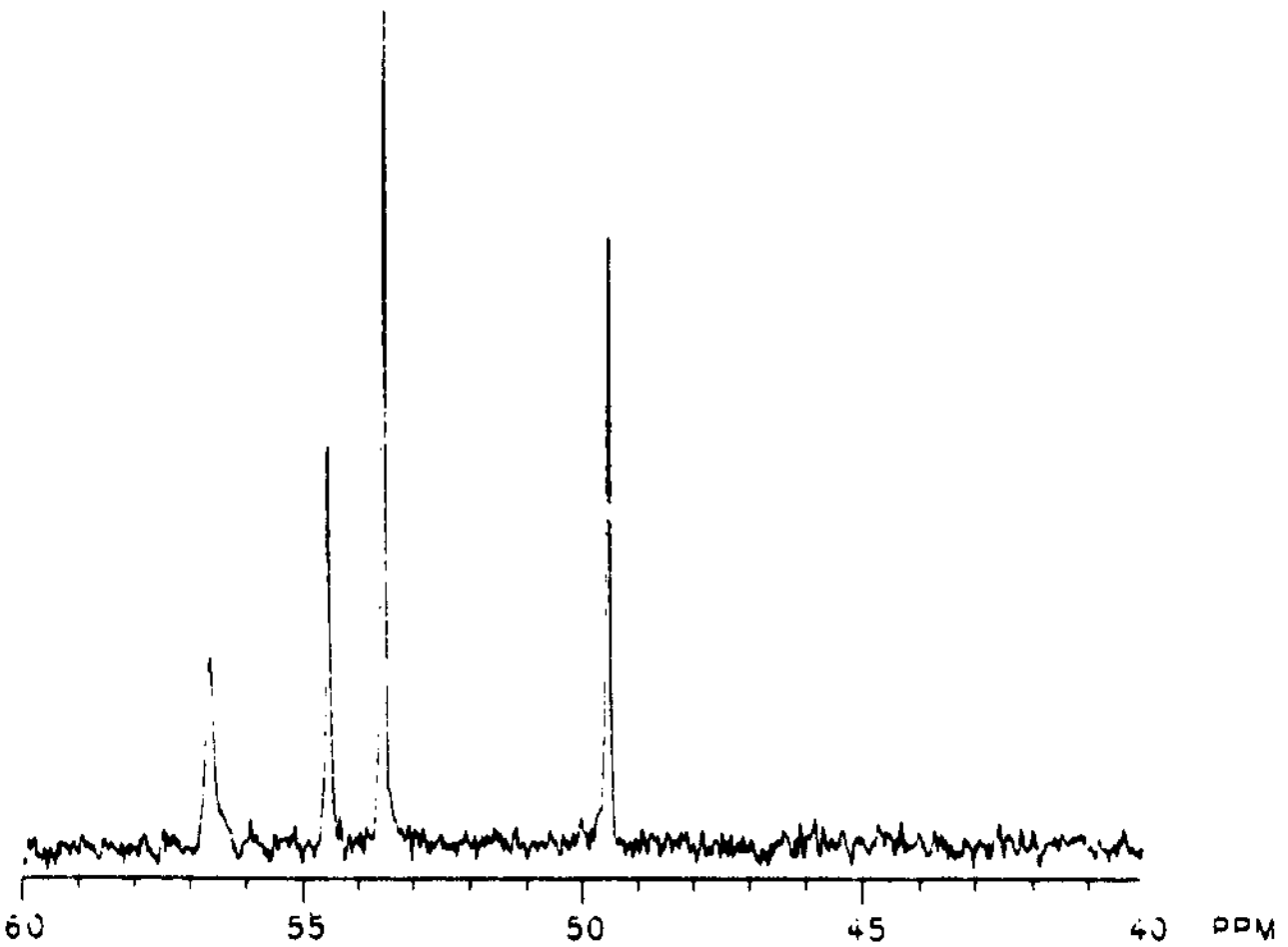
The results of a single addition experiment using VBI of DMPC and exogenously added $YbCl_3$ are shown beginning in Figure 34. The vesicle are composed completely of choline [N-$^{13}CH_3$]-

Figure 34 Carbon-13 NMR Spectra of N-Methyl Choline-Labeled DMPC: Vesicles by Injection. The lower spectrum (A2) is of an homogeneous preparation of VBI of isotopically pure [choline, N-$^{13}CH_3$] DMPC prepared in Na-PIPES according to the standard method and diluted to 1.3 mM. The upper spectrum (A1) is buffer alone. Instrumental parameters were as described in Methods. 1000 acquisitions were averaged. Signals reflect 2 Hz line-broadening. Trace widths are approximately 9 KHz (100 ppm). Note that the resonance in spectrum A2 at $\delta \approx 56.6$ ppm is absent in spectrum A1. See the text for details.

A1 BUFFER



A2.



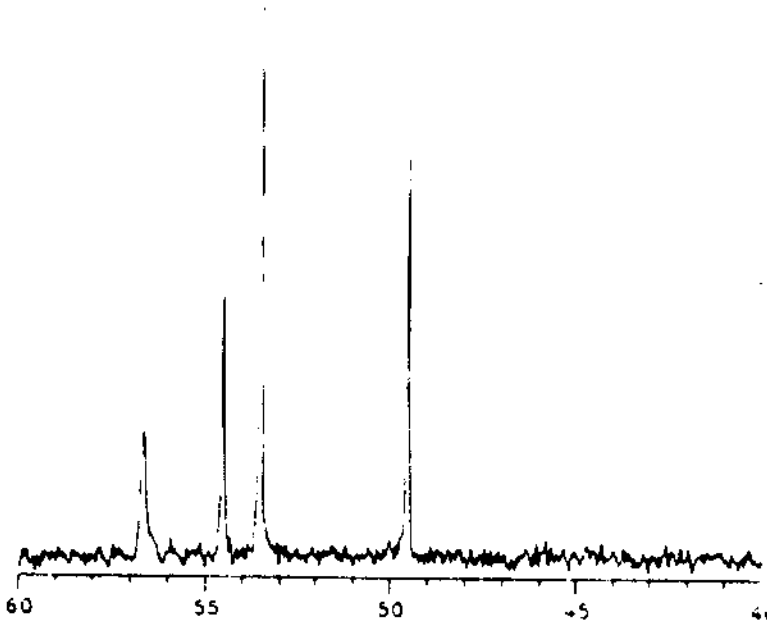
enriched DMPC; consequently, the required concentration is less than 2 mM. Of the four resonance lines seen, three correspond to carbon atoms of buffer molecules. It is to these resonances that the spectrum is referenced (18). The upper trace of Figure 34 presents the buffer spectrum, the lower VBI in buffer. A single line is observed for the vesicles from this 30 min. experiment. The lower trace of Figure 34 is shown with its expansion in Figure 35. Also shown is the spectrum and expansion of this vesicle preparation after addition of a single aliquot of Yb^{3+} to a metal-to-phospholipid ratio of 0.080. Although the experiment was ended before performing a sufficient number of acquisitions to give accepatable signal-to-noise ratio, a second resonance is suggested in the expansion. The poor signal-to-noise and the lack of resolution of the two resonances in this 30 min. experiment suggest that vesicle integrity may have changed during the course of the experiment.

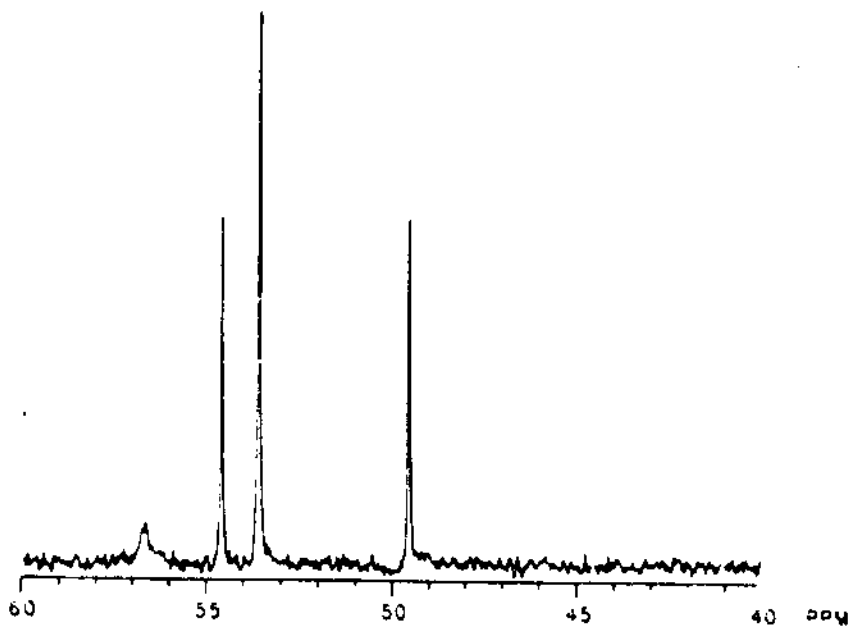
G. Fluorescence Polarization Measurements in PC and PC/PA Vesicles

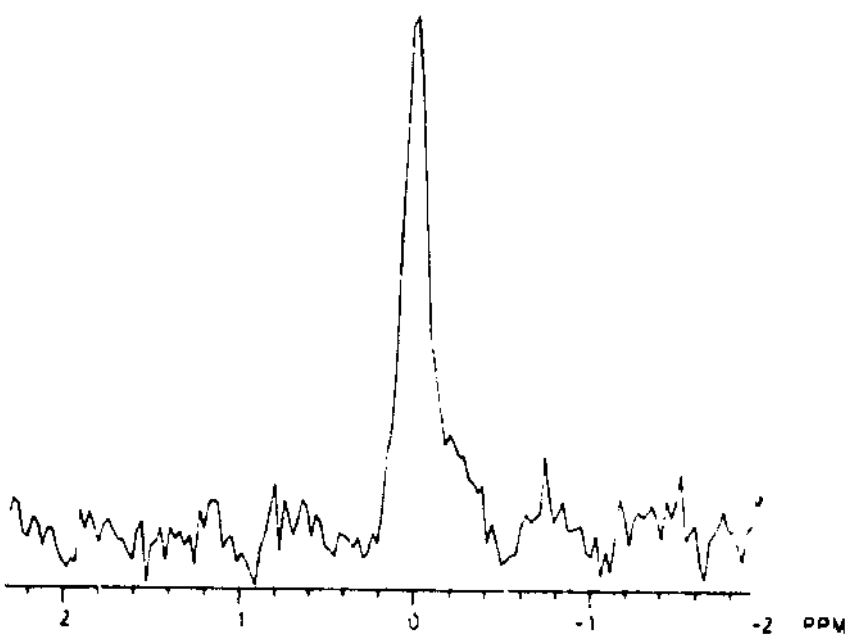
The aims of this project as a whole were two: to produce a model membrane system in which a phospholipid asymmetry had been induced and to determine the effect of the asymmetry on the dynamic properties of the membrane. Preceding sections have described efforts directed toward the first aim: (i) development of a standard method to prepare and characterize vesicles ≈90 nm in diameter, (ii) experiments suggesting that phospholipase D could be used as a means to hydrolyze up to ≈50% of the outer leaflet of PC vesicles to PA, even in the absence of Ca^{2+} and (iii) NMR studies establishing experimental conditions which allow resolution and quantitation of outer and inner leaflet resonances of vesicles.

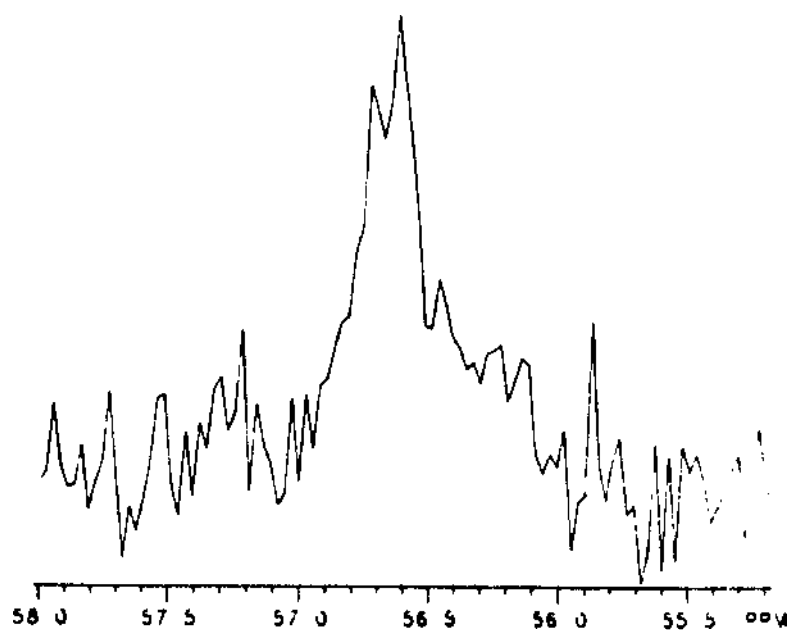
Of the wide variety of methods available for determining effects of perturbations on the dynamic properties of membranes (see Introduction), fluorescence spectroscopic methods are highly sensitive and therefore requirement of very low levels of exogenous probe; moreover, they offer

Figure 35 Carbon-13 NMR Spectra of N-Methyl Choline-Labeled DMPC: Vesicles by Injection, with Added Ytterbium. Spectra shown are of the preparation of VBI described in the preceding figure, with and without added $YbCl_3$. The spectrum in the upper left, A2, is identical to spectrum A2 in the preceding figure. The spectrum in the lower left (A2ex) is a 5-fold expansion of spectrum A2 about the VBI resonance. The spectrum in the upper right (B2) was acquired after the buffer on the outside had been exchanged with an isotonic buffer (in the manner describe in the legend to Figure 33) such that the ytterbium-to-phospholipid ratio = $[Yb^{3+}]$ / [DMPC] = 0.080. The spectrum in the lower right (B2ex) is an 8-fold expansion of spectrum B2 about the VBI resonance. All signals reflect 2 Hz line-broadening. Trace widths for A2 and B2, and A2ex and B2ex are 1.8 KHz (20 ppm), and 360 Hz (4 ppm) and 225 Hz (2.5 ppm), respectively.


60
55
50
45
40
ppm


60
55
50
45
40
ppm


2
1
0
-1
-2
ppm


58 0
57 5
57 0
56 5
56 0
55 5
ppm

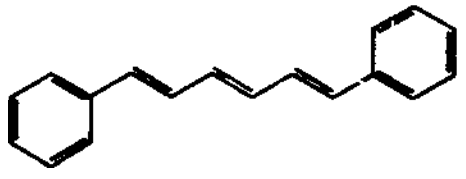
the opportunity to observe dynamic processes on the nanosecond time-scale (91, 107, 199, 198, 62). Of the number of fluorescence methods available for the study of the dynamics of membrane properties, the measurement of the polarization (or anisotropy) of the emission of membrane-localized flurophores provides a simple means for estimating such parameters as the internal viscosities of membranes and for studying the effect of changes in membrane structure on the phase behavior of the membrane (197, 108).

The structures of a number of fluorescent probes of interest are shown in Figure 36. N-NBD-PE was used in the HPLC characterization of VBS and VBI described above. The probes 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-[4-[(trimethylammonio)phenyl]-6-phenyl-1,3,5-hexatriene (TMA-DPH) were selected to characterize the asymmetric model systems based on the regions of the membrane that they probe and on the sensitivity of their polarization to local lipid environment (170, 8, 63, 37, 141).[1] Also shown are two of the dipole moment-sensitive 6-acyl-2-dimethylaminonaphthalene (prodan) derivatives of Weber and co-workers (119, 200): the somewhat amphipathic 6-lauroyl derivative, laurodan, which is thought to be anchored in the headgroup-glycerol region by the tertiary amino group, and a proposed glucosamino derivative of 6-lauroyl-2-methoxynaphthalene (laurmen), whose synthesis is outlined at the close of this section.

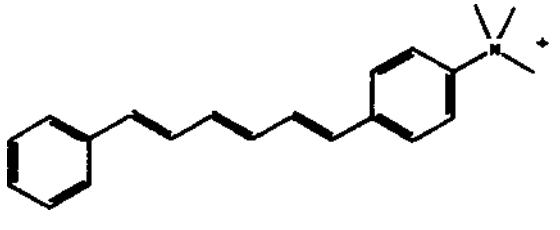
The first set of fluorescence experiments was designed to compare the temperature dependence of the polarization of DPH and TMA-DPH in phospholipid vesicles. DPH is thought to localize in the hydrocarbon milieu, with its C_2 axis perpendicular to the plane of the bilayer of membranes in the gel state (37, 140). Its utility lies in the sensitivity of its polarization to even small changes in its

[1]TMA-DPH was initially chosen over such amphipathic probes as the 4-acyl-7-hydroxycoumarins of Pal *et al.* (136), the oligosaccharide- and glutathione-linked pyrenes of the laboratory of Schachter (cf. 34), the dialkyl indocarbocyanines of Wolf (205) and the dipole moment-sensitive 6-acyl-2-dimethylaminonaphthalene (prodan) derivatives of Weber and co-workers (119, 200), primarily for purposes of comparison with DPH.

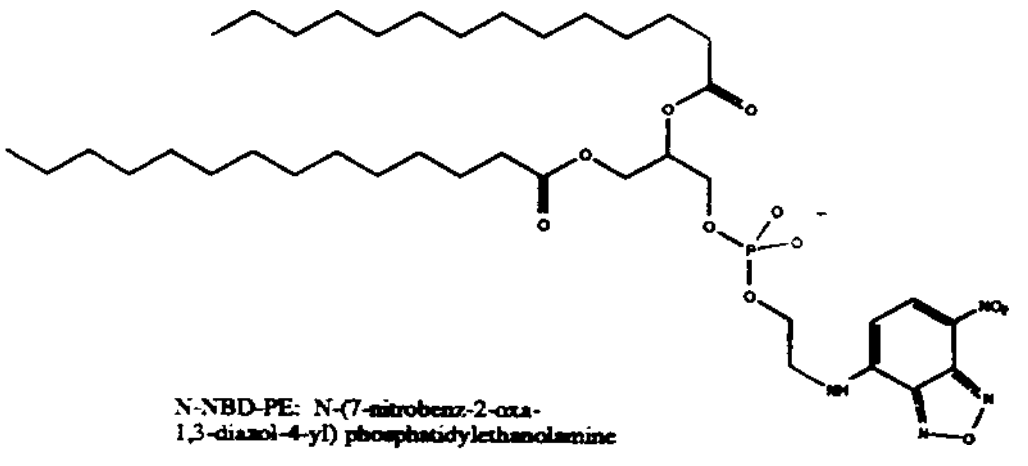
Figure 36 Structures of Hydrophobic and Amphipathic Fluorescent Probes of Interest. The structures shown include the N-NBD-PE probe used earlier for phospholipid detection during HPLC, and hydrophobic DPH and TMA-DPH probes used in steady-state depolarization measurements. A second amphipathic, cationic probe is "patman", a dipole moment-sensitive probe in the family of 2,6-disubstituted naphthalenes introduced by Weber and co-workers (200). Also in this family is a group of proposed amphipathic, neutral probes based on schemes linking a glycosyl moiety to precursors of the Weber probes laurodan (6-lauroyl-2-dimethylaminonaphthalene) and lauromen (6-lauroyl-2-methoxynaphthalene). One example of the proposed family is shown in the lower right hand corner of the Figure.



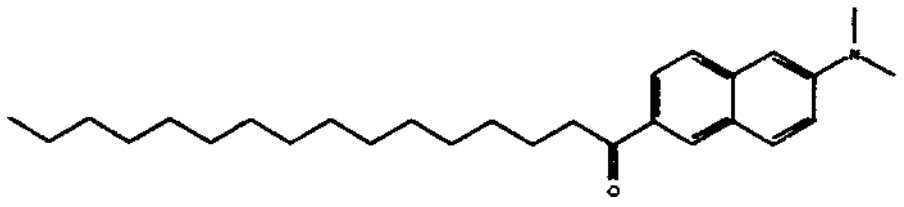
DPH: 1,6-diphenyl-1,3,5-hexatriene



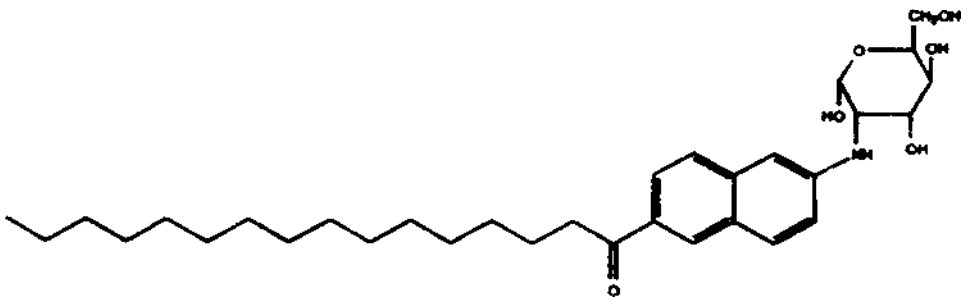
TMA-DPH: 1-[4-(trimethylammonio) phenyl]-6-diphenyl-1,3,5-hexatriene



N-NBD-PE: N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) phosphatidylethanolamine



Laurodan: 6-palmitoyl-2-dimethylaminonaphthalene



And a player to be named named later: a proposed glucosamino relative of laurdan

Hydrophobic and Amphipathic Fluorescent Probes of Interest

immediate lipid environment and in the wealth of data available for comparison available (170, 171, 8, 63). TMA-DPH was originally designed by Prendergast *et al.* to "anchor" DPH at the bilayer-aqueous interface, yet to allow a significant portion of the fluorophore to penetrate to the hydrocarbon milieu (141). These authors report that "while its photophysical properties are fundamentally similar, the patterns of motion and, by inference, the region of the bilayer reported on by TMA-DPH are quite different from that of the parent molecule."

Parallel experiments were performed on standard preparations of vesicles prepared by sonication and (VBS) by injection (VBI) of egg yolk PC and of 30 mol% egg yolk PA in total egg yolk phospholipid (PA + PC). An aliquot of each of the four preparations of vesicles were labeled with either DPH or TMA-DPH. Polarization measurements were then performed on these eight samples at a small number of temperatures between 0 and 50°C. Polarization values at the low and high temperatures were compared and rates of change of measured polarization with temperature were estimated by curve fitting. Pair-wise comparisons of the results for each unique set of conditions (probe, vesicle diameter, vesicle composition) were performed with the aid of logic diagrams developed for complex circuit analysis (92, 192).

The diagram in Figures 37 presents data from a representative experiment, where polarization values were recorded at 3°C for each sample. The following observations are based on the pairwise comparison of polarization values for samples differing in either probe, vesicle diameter or composition. (i) As expected, values of polarization for TMA-DPH are consistently higher than DPH for vesicles of the same diameter and composition (141). The greatest difference between TMA-DPH and DPH were noted for VBS of PC, the least for VBI of PC. (ii) In addition, values of polarization for either probe in PC/PA vesicles are consistently higher than in vesicles of pure PC of the same diameter, though only negligibly so in the case of VBS probed with TMA-DPH. In contrast, the most significant difference between PC/PA and PC vesicles was noted in the case

Figure 37 DPH and TMA-DPH Fluorescence Polarization Measurements in PC and PC/PA Vesicles: Polarization at 3°C. Homogeneous preparations of VBS and VBI were prepared from egg yolk PC and from egg yolk PC containing 30 mol% egg yolk PA. Probe was addition and concentration was as described in Methods. Final phospholipid concentration was approximately 0.2 mM in Na-PIPES. Polarization values were calculated from data acquired on an SLM 4000 spectrofluorimeter; excitation and emission wavelengths were 360 and 430 nm, respectively. For further procedural detail, see Methods. Presentation of the data is an adaptation of the tables of W.E. Veitch (192) and M. Kamaugh (92). The ends of the tabbed rectangle are conceptually joined at the tabs to form a cylinder, and the "table" that results presents all comparable data in adjacent positions. That is, each polarization value can be validly compared only with values immediately to the left, right, above or below it. See the text for interpretation of the data.

Fluorescence Polarization of Vesicles at 3° C

	Phosphatidylcholine		Phosphatidylcholine /Phosphatidic Acid	
	VBS	VBI	VBI	VBS
DPH	0.210	0.236	0.273	0.237
TMA-DPH	0.347	0.305	0.380	0.350

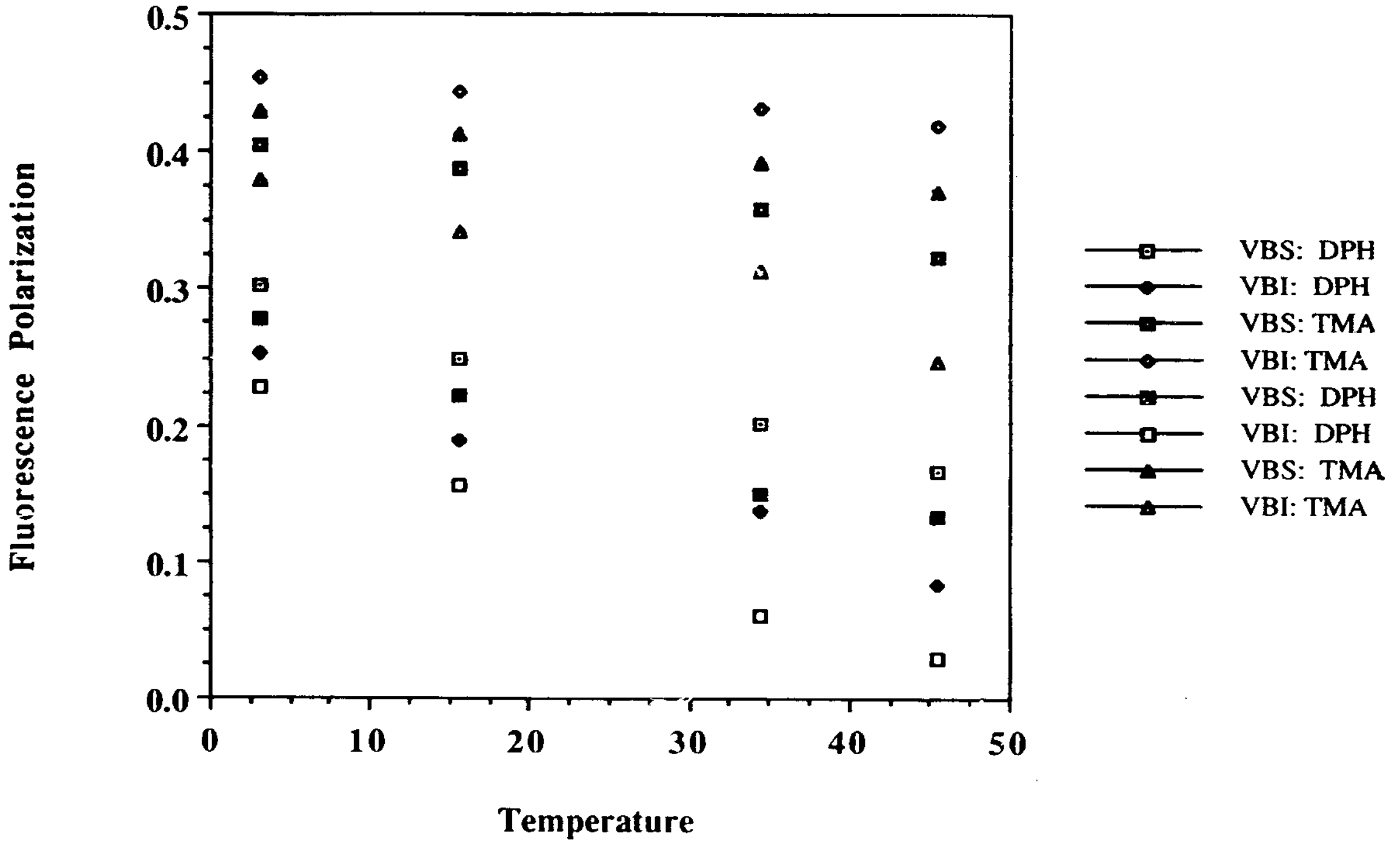
of VBI probed with TMA-DPH. (iii) Finally, values of polarization for either probe in the 90 nm VBI are consistently higher than in the minimal diameter VBS of the same composition; the magnitude of the difference is ≈0.03 units. The striking exception is the case of pure PC vesicles probed with TMA-DPH, where the polarization for VBS is ≈0.03 units higher than for VBI.

In Figures 38 data are presented for an experiment where polarizations were recorded at increasing temperatures for each of the eight samples. The following observations are based on the pairwise comparison of the estimated changes in polarization with temperature for samples differing in either probe, vesicle diameter or composition. (i) Changes in DPH polarization are always greater than the changes observed for TMA-DPH for vesicles of the same diameter and composition. (ii) The change in polarization with temperature noted for pure PC vesicles are equal to or greater than those noted in PC/PA vesicles when vesicles are small, but are less than or equal when vesicles are large. The greatest headgroup specific changes were noted with TMA-DPH as a probe. (iii) The changes noted for VBI are greater than for VBS for either probe in PA/PC vesicles, are equal in DPH-probed pure PC vesicles and are less than in TMA-DPH-probed pure PC vesicles.

When interpreted in light of the known asymmetry of PA in VBS of PC/PA, the known asymmetry in packing in VBS in general (150), the expected symmetry of both of these in VBI (131) and the expected regions probed by DPH and TMA-DPH, the following tentative conclusions are offered. (i) While DPH is a more sensitive probe of temperature-dependent environmental changes, it is generally insensitive to the structural and dynamic differences seen by TMA-DPH in PA/PC vesicles, i.e., to effects nearer to headgroup. (ii) It appears that when PA is primarily in the inner leaflet in a vesicle with packing constraints (e.g., VBS), it serves to rigidize the membrane. In the symmetric non-constrained case (VBI), however, PA serves to fluidize the membrane, consistent with limited published information (141). (iii) The effect of the presence of PA is generally more

Figure 38 DPH and TMA-DPH Fluorescence Polarization Measurements in PC and PC/PA Vesicles: Changes in Polarization with Temperature. Homogeneous preparations of egg yolk PC and PC/PA vesicles were prepared and labeled as described in the preceding Figur . Polarization values were determined from data acquired as described, at four temperatures from 4° to 46° C. Samples were equilibrated for ≈15 min. at each temperature prior to making measurements. The curves reflect the change in measured polarization with increasing temperature. Each curve is offset by the addition of a constant (to each polarization value within the set) so that the degree of change of polarization with temperature relative to other sets is apparent. Actual polarization values cannot be read from the ordinate; see the preceding figure for polarization values measured for each set at 4° C. See Methods and the text for more information.

Rotational Mobilities of Probes in Vesicle Membranes



pronounced in large diameter vesicles than in small. (iv) The headgroup region of the outer leaflet of VBS of pure PC appears to be more fluid than that of VBI of pure PC.

G. The Potential of Prodan-based Fluorophores as Probes of Asymmetric Vesicles

The 2,6-disubstituted naphthalenes of Weber and co-workers (119, 200) were considered as synthetic precursors for an amphipathic membrane probe with a neutral rather than charged anchoring moiety. Fluorescence emission spectra of laurodan, the 6-lauroyl analog of prodan, were measured in a variety of solvents (Figure 39). Emission maxima were found to be equally sensitive to solvent polarity, and slightly blue-shifted relative to prodan (Table 13). Thought was given to coupling this equally sensitive lauroyl derivative to a glycosyl moiety in the 2 position. Discussions with Dr. Gerard Merriott lead to the following suggestions for syntheses of such probes: (i) addition of a glucosamine to laurmen in the presence of Li/hexamethylphosphoric triamide/benzene, resulting in the structure shown in Figure 36, (ii) condensation of glucuronic acid and 6-lauroyl-2-naphthoic acid with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide/N,N-dichlorourethane in pyridine/dimethylformamide (1:1), (iii) addition of 6-hydroxy-2-naphthoic acid sequentially with SO_2Cl and dodecylamine, followed by addition of a glucosamine in the presence of $NaHSO_3$, and (iv) synthesis of the sulfonyl chloride of 6-palmitylamino-2-naphthalene sulfonic acid with PCl_5, followed by addition of a glucosamine. The addition of various diaminoalkanes to 6-propionyl-2-methoxynaphthalene (promen) in the presence of Li/hexamethylphosphoric triamide, followed by the addition of the acid chloride of glucuronic acid would make available a number of probes differing in the length by which the naphthyl moiety penetrates the bilayer.

Figure 39 Fluorescence Emission Spectra of Laurodan in Solvents of Varying Polarity. Emission spectra (excitation wavelength, 360 nm) of laurodan [6-dodecanoyl-2-dimethylamino-naphthalene] were recorded for a series of solvents of varying polarity. A small aliquot of a room temperature saturated solution of probe in methanol was dried and redissolved in high purity solvents: A - cyclohexane; B - chlorobenzene; C - dimethylformamide, D - ethanol and E - methanol. The concentration at this 1:400 dilution of the stock corresponded to an absorbance at 360 nm of approximately 0.06 AU. Further information on equipment and data processing are described in Materials and Methods.

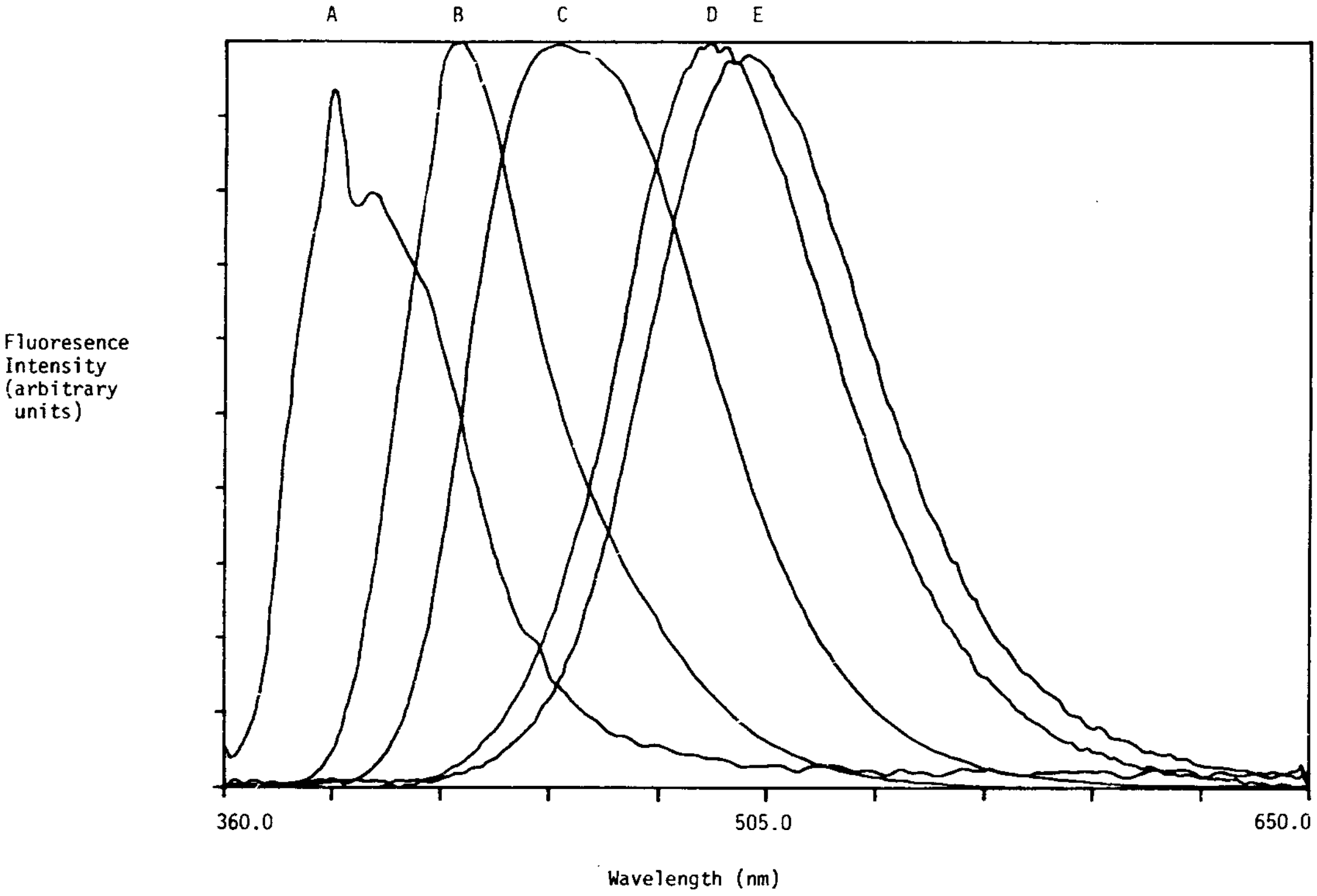

A
B
C
D
E
Fluoresence Intensity (arbitrary units)
360.0
505.0
650.0
Wavelength (nm)

Table 13 Comparison of Spectral Shifts of 6-Acyl-2-dimethylaminonaphthalenes in Various Solvents

Solvent	Emission maxima (nm)	
	Prodan[a] [6-propionyl-]	Laurodan[a] [6-lauroyl-]
cyclohexane	401	400
chlorobenzene	430	424
N,N-dimethylformamide	461	451
ethanol	496	489
methanol	505	500
water	531	--

[a]Data from Weber and Farris (for prodan, ref.200) and this work.

V. Conclusions and Recommendations for Future Research

The purpose of the work proposed was to develop a means for producing and characterizing large-diameter phosphatidylcholine (PC) vesicles with a significant outer leaflet concentration of phosphatidic acid (PA), and to assess the changes in the physical properties of the vesicle membrane imparted by the asymmetric distribution of PA. Production necessarily included steps to form the vesicles, to induce the asymmetry, to remove the agents used to disperse the lipid and generate the asymmetry, and to separate unilamellar vesicles from multilammelar and unsealed structures. Characterization necessarily included determining the mean diameter and the number of lamellae per vesicle of the vesicle population, demonstrating that the vesicle membrane was sealed and measuring the outer-to-inner leaflet ratios of each species of phospholipid. The assessment of physical properties was to be directed toward determining changes in the rotational mobility of appropriate probe molecules by steady-state flurescence polarization measurements.

A. Methods to Produce Large Diameter Vesicles

The method developed to produce crude preparations of large-diameter vesicles was based on the work of Nordlund *et al*. (131), Kremer *et al*. (103) and others. Controlled injection of ethanolic

phospholipid was accomplished using a commercially-available metered syringe pump (described in Methods). The stirred, neutral encapsulation buffer was held at a temperature above the T_c of the highest melting phospholipid by an ordinary thermostated bath. The method proved to be a simple, efficient and reproducible way to produce large-diameter phosphatidylcholine vesicles. As product vesicle diameter varies with the concentration of injected lipid, the method has further potential in experiments designed to determine the dependence of lipid asymmetry (or of specific physical properties) on membrane curvature.

The use of degassed buffers, an inert atmosphere and metal ion chelators prevented oxidation of the unsaturated egg phosphatides used throughout this work. The necessity of such precautions is evident from studies noting the effect of the accumulation of lipid oxidation products on phospholipid symmetry and on physical measurements in general (169, 187). The spectrophotometric method used to assess the degree of decomposition was simple and rapid, encouraging its continued use (88). The thin layer chromatographic method used to characterize the purity of phospholipids throughout the experiments were simple and reliable (156), but tedious when quantitation was desired. Consideration was given to applying rapid, high performance liquid chromatographic methods, and specifically to the problem of phopholipid detection (184).

Essentially all injected ethanol could be removed by a single, rapid passage of the crude vesicle population over a column of Sephadex G25. The gel filtration method (cf. 116) was therefore routinely applied for the removal of ethanol and for changing the buffer to which outer leaflet phospholipid was exposed. The concentration of vesicle phospholipid prior to gel filtration was low enough (≈10 mM) to require an added preparative step when vesicles were prepared for ^{31}P nuclear magnetic resonance (NMR) experiments. When vesicles were prepared for fluorescence measurements, phospholipid concentrations were sufficient even after gel filtration. Crude preparations of vesicles were routinely concentrated after gel filtration using a commercially

available vacuum dialysis unit (described in Methods), a method which was found to be more efficient than traditional concentration methods (180).

The simple and rapid preparative ultracentrifugation methods developed in the laboratory of T.E. Thompson (cf. refs. 10, 17) were adapted to separate unilammelar vesicles from multilammelar vesicles. Extrusion methods were also considered for further increasing the homogeneity of vesicle preparations with respect to diameter. Data from experiments (summarized below) provided evidence that the ethanol-free vacuum dialyzed vesicles were essentially a single distribution about a mean diameter of ≈90 nm. The distribution was narrowed somewhat by a short period of centrifugation; this step was therefore retained. The calculated outer-to-inner phosphatidylcholine ratio (116) based on a 90 nm diameter and the assumption that vesicles were unilammelar matched the experimental ratio determined from ^{31}P NMR spectra acquired in the presence of shift reagent (see below). These preparative steps were therefore deemed sufficient for routine use in producing a population of large-diameter, unilamellar vesicles. Extrusion methods and other preparative steps were omitted.

B. Assessing Chemical Purity, Diameter and Dispersity of Vesicles

The method used to characterize the dispersity and mean diameter of vesicle preparations was the Sephacryl S1000 gel filtration technique of Reynolds, Tanford and co-workers (153), with a standard curve calculated using the inverse error function (2) as applied by Ackers (3, 4). There are a number of practical difficulties associated with the Sephacryl S1000 method. High back-pressures are observed after the Sephacryl is pretreated with phospholipid to minimize non-specific lipid binding. Multiple characterizations of void and total column volumes (V_o and V_t, respectively) are necessary prior to chromatographing unknowns because of changes in gel volume

upon (i) pretreatment with phospholipid, and (ii) chromatography in the sodium dodecyl sulfate-containing elution buffers required for latex diameter standards.

Routine use of this method would require high quality analytical chromatography hardware to ensure the reproducibility of the low flow rates required for analytical gel filtration at the high back-pressures observed with phospholipid pre-treated columns. With such equipment, initial packing, chromatographic analysis of standards (calibration), phospholipid pre-treatment and recalibration could be performed over a period of approximately 2-3 weeks. Each chromatographic analysis of unknowns could then be performed over 1-2 days. These practical concerns suggest that consideration be given to alternatives to the Sephacryl S1000 gel filtration method. Among those worth consideration are high performance gel filtration methods (134) and quasielastic light scattering methods (64), the latter of which are now accessible through commercially available instruments. Preliminary results from vesicles chromatographed on a high performance TSK GW6000 gel filtration column were in accord with Sephacryl results.

C. Purification of Phospholipase D

Phospholipid asymmetry was induced in populations of the 90 nm vesicles by exposure to preparations of phosphatidylcholine phosphohydrolase (phospholipase D) from a commercial source (described in Methods) or purified from Savoy cabbage (cf. Clancy, ref. 31). In the purification of the phospholipase from cabbage, added hydrophobic affinity and gel filtration steps were considered. Evidence suggests the former as potential step in future purification of the enzyme. The Sephadex G200 gel filtration step was once again added as a routine purification step (31, 5) as required by the use of G200 at a later point in the experimental scheme to separate enzyme and free choline from product PC/PA vesicles. These and other column chromatographic

steps would be more efficiently performed if the high quality chromatography hardware noted above were available.

Enzyme purified through the γ-aminopropane-agarose affinity step (5, 31) was shown to consist of 5-20% phospholipase D (by polyacrylamide gel electrophoresis, based on the established M_r of the enzyme) and a number of low molecular weight contaminents, possibly of proteolytic origin. In the same experiment a loading of an equal mass of the commercial preparation of enzyme was observed to be surprisingly free of low molecular weight contaminents. The necessity of removing glycol and sugar stabilizers prior to the use of either enzyme preparation was noted, in light of the alcohol transferase activity of the enzyme.

D. PHLD Conversion of PC to PA

Early work with the commercial enzyme demonstrated that intact PC/PA vesicles could be generated from vesicles by sonication hydrolyzed in the presence of calcium. In the studies that followed an attempt was made to characterize the activity of different enzyme preparations toward three phosphatidylcholine substrate forms: (i) PC in an ether-water biphase, (ii) PC in dispersions (multilamellar vesicles) and (iii) PC in 90 nm unilamellar vesicles. Enzyme preparations at different stages of purification were routinely found to differ markedly in the characteristic pH and calcium concentration required for optimal hydrolysis of a particular form of substrate, a result consistent with the variability of published data in the field.

Results were in general accord, however, with Allgyer and Wells' report of two [Ca^{2+}]-dependent pH optima for the enzyme (5), and with DeKruijff and Backen's report of the activity of the enzyme toward vesicle substrates even in the absence of calcium (43). In addition, the following two general features were were observed. First, PC in 90 nm vesicles prepared by injection was

always an equally good or better substrate than PC in biphase or dispersions. Second, the percent conversion of PC to PA increased as expected with extended time, though in a manner disproportionate to the initial rapid rate. Possible explanations were offered for these observations.

An experiment comparing the extraction of choline from reaction mixes suggested that the ether-water method of Clancy (31) allowed accurate calculation of the extent of phospholipase D reaction. This observation led to the the design of a simple method for unambiguously measuring PC/PA ratios in reaction mixes with both [choline, N-C^3H_3]- and [1-acyl, 1-^{14}C]- labeled phospholipids, where PA production was quantitated by following the decrease in the specific activity of the ^3H label relative to the ^{14}C.

Using this method, a commercial preparation of phospholipase D was shown to catalyze the hydrolysis of ≈30 mol% of the total PC of 90 nm vesicles by injection during a 90 minute incubation at pH 6.0 in the absence of calcium. Calculated (116) or measured (see below) outer-to-inner leaflet ratios suggest an outer leaflet concentration of 50 mol% PA if the mean vesicle diameter remained unchanged, and no translocation (flip-flop) disturbed the created asymmetry. No increase in turbidity or other evidence of vesicle aggregation or precipitation was observed. Preliminary attempts were made to characterize the vesicles using ^{31}P NMR (see below).

The successful use of phospholipase D to generate PC/PA vesicles encourages its continued use as a means of inducing a phospholipid asymmetry in model systems. It is likely that the more general and, in some cases, separable alcohol transferase activity (90, 182) can be similarly applied to produce other phospholipid asymmetries. The number of reported phospholipase and related transferase activities continues to expand, providing possible alternatives to the use of the cabbage enzyme. The appropriate choice of the source of the activity and the application of what are now

standard methods for producing and over-expressing recombinant proteins (cf. 114) may allow one to circumvent the problems of low activity in the absence of calcium, low overall yield of purification and seasonal variation in quality characteristic of the enzyme isolated from cabbage.

E. Analysis of the Asymmetry of Phospholipids in Vesicles using NMR

Methods to demonstrate that vesicle membranes of 90 nm vesicles (and accordingly the product PC/PA vesicles) were sealed, and methods to determine outer-to-inner leaflet ratios of phospholipids were based on the quantitative ^{31}P NMR measurements introduced L.D. Bergleson, L.I. Barsukov and co-workers (194, 16) and developed in their laboratories and in the hands of W.C. Hutton, P.L. Yeagle and R.B. Martin (84). The measurements depend on a correspondence between an observed resonance intensity and the number of nuclei giving rise to the resonance, a condition obscured by nuclear Overhauser effects (NOE) and other relaxation-related phenomena.

A Waltz gated proton decoupling sequence based on the early report of Freeman *et al.* (58) was successfully used to eliminate $^{31}P\{^{1}H\}$ NOE enhancements. The sequence was evaluated on the model compounds phosphorous acid trimethyl ester, glycerophosphate and phosphoryl-ethanolamine. Application of the gated decoupling technique at the same field strength of earlier published measurements yielded an NOE enhancement for the phosphorous acid ester that was essentially equal to the published value (212). The measurement performed on glycerophosphate at an ≈2.5 fold higher field strength was attenuated by ≈40%, in accord with the theoretical prediction of NOE field strength dependence (208, 209).

This significant attentuation suggested that shorter recycle times could be used without sacrificing quantitation. The NOE enhancement determined for phosphorylethanolamine using a shorter

recycle time (only slightly greater than its approximate T_1) was comparable to enhancements measured for similar molecules measured with longer recycle times. Appropriate levels of decoupler power were also determined during these experiments. Similar measurements were perfomed on vesicles formed by sonication of dimyristoyl PC and egg yolk PC, and on 90 nm vesicles formed by injection of egg yolk PC. Analysis of the NOE enhancements for each of these vesicles in light of the 40% attenuation of NOE with field strength noted for glycerophosphate suggested that they were either exactly comparable to or slightly lower than published values for these systems. On the whole, the results were judged to be close enough to expected values to proceed to experiments using shift reagents with standard vesicle systems.

Results from ^{31}P NMR experiments involving titration of sonicated vesicle populations with the line-shift reagent praesodymium (III) chloride were consistent with expectations based on published accounts of the method (16, 84). Complete separation of the outer and inner leaflet PC resonances was achieved at $[Pr^{3+}]/[\text{phospholipid}] \approx 0.05$. A correspondence was noted between the point at which the slope of the titration curve ($\Delta\delta$ vs. $[Pr^{3+}]/[\text{DMPC}]$) changes and the transition in mechanism from line-shifting to line-broadening for the Pr^{3+} reagent. This result is discussed. Significantly, the measured ratio of outer leaflet PC to inner leaflet PC for sonicated vesicles was exactly that expected based on vesicle diameter (84, 116). The recycle times in use were therefore considered acceptable for quantitating outer-to-inner leaflet phospholipid ratios.

The same required stoichiometry ($[Pr^{3+}]/[\text{phospholipid}] \approx 0.05$) was noted for 90 nm vesicles prepared by injection. No differences were noted between experiments where Pr^{3+} was added exogenously and experiments where buffer in contact with the outer leaflet of vesicles was exchanged with Pr^{3+} containing buffers by vacuum dialysis or gel filtration. Vesicles formed in the presence of shift reagent and dialyzed or chromatographed against buffers without shift reagent

confirmed the essentially unilamellar nature of the vesicles. Also, spectra acquired intermittently over 24 hrs. testified to the stability of the vesicles when preparations were stored at a temperature above the T_c of the PC from which they were formed. Results indicated that the barrier properties of the membrane remained intact for periods longer than typical experimental time-frame.

The difference in the chemical shifts of PC and PA was observed to be ≈80 Hz, and the difference between fully shifted outer and inner leaflet PC was ≈ 30 Hz. It was therefore considered probable that the vesicle product of the hydrolysis of double-labeled 90 nm vesicles (described above) would at least initially give rise to separate resonances upon exposure to Pr^{3+}, allowing quantitation of outer leaflet PA and of PC in both leaflets.

Preliminary NMR experiments on asymmetric sonicated PC/PA vesicles demonstrated that exposure of the PA containing leaflet to free Pr^{3+} anion results in undesirable effects on line shape and chemical shift.[1] The result suggested that experiments to determine the ratios of PC and PA of the inner and outer leaflets in asymmetric vesicles formed by phospholipase D treatment might be best performed with encapsulated rather than exogenously-added shift reagent. The exact conditions for quantitative work with the PC/PA system remain to be determined.

If encapsulation fails to allow quantitative determination of PC and PA in each leaflet, cations could be offered to the exposed leaflet in complexes, perhaps minimizing binding events giving rise to the spurious results noted. In the event that a single shift or line-broadening reagent proves ineffective, a series of titration experiments using two or more reagents could be designed (16). In

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[1]In this case, results differed from published observations of the effect of a paramagnetic ion on asymmetric PC/PA vesicles prepared by sonication an phospholipase D treatment (43), where external exposure to Mn^{2+} resulted in the desired line-broadening. This published result needs to be considered critically, however, in light of the ability of Mn^{2+} to catalyze transbilayer redistribution of phosphatidyl glycerol, another acidic phospholipid, in PC/PG sonicated vesicles (113).

addition, the PA concentration-dependence of paramagnetic ion-induced chemical shifts could be evaluated as a means of quantitating PA. Using this method, the concentration of phoshatidylinositol (PI) was determined over a range of 1-30% of PI in PC by ^{31}P NMR in the presence of Yb^{3+} (17, and ref. 9 therein).

E. Measurements of the Dynamic Properties of Membranes

Fluorescence polarization measurements were directed toward determining the effect of an asymmetric distribution of phospholipid on the dynamic properties of vesicle membranes (197, 108). While only fluorescence methods will be referred to here, the availability of higher field strengths and new relaxation-based techniques makes NMR an attractive alternative for future projects characterizing the dynamic properties of phospholipids in membranes (cf. 46, 54, 23, 95, 176). When applicable, NMR methods offer dynamic information over a broad range of frequencies (10^{-1} - 10^{10} sec^{-1}, ref. 62), and often are performed at natural abundance levels, using stable isotopes native to the structure being studied. Addition of exogenous probes (with the added concerns that they bring, ref.198) are therefore unnecessary.

Preliminary fluorescence experiments performed suggest that small vesicle diameter and an asymmetry in the distribution of PA in PC/PA vesicles effected the rotational mobility of probe molecules localized in two regions of the vesicle bilayer. Rotational mobilities were inferred from steady-state flurescence polarization measurements performed using the probes 1,6-diphenyl-1,3,5-hexatriene (DPH, refs. 54, 108, 141), a probe of the core of the fatty acyl region of the bilayer, and 1-[4-[(trimethylammonio)-phenyl]-6-phenyl-1,3,5-hexatriene (TMA-DPH, ref. 141), a probe of the fatty acyl region near the bilayer-aqueous interface.

Parallel experiments were performed on standard preparations of vesicles prepared by sonication or ethanol injection of egg yolk PC or of 30 mol% egg yolk PA in total egg yolk phospholipid (PA + PC) that had been labeled with either DPH or TMA-DPH. The measured polarization at 4°C and the estimated change in polarization with temperature over a range of 0-50°C was reported for each unique combination of probe, vesicle diameter and vesicle composition. Observations based on pairwise comparisons of the changes in polarization were simplified by a novel use of logic tables (92), and were interpreted in light of (i) the reported regions probed by DPH and TMA-DPH, (ii) the known difference in the diameters of sonicated and injected vesicles (≈22 vs. ≈90 nm, respectively) and (iii) the reported (14, 39, 90) and presumed (131) asymmetry of PC/PA sonicated and injected vesicles, respectively. Data and interpretations, while tentative, were seen to be consistent with the limited published information on these vesicle systems (14, 39, 90, 141).

DPH was noted to be the more sensitive probe of temperature-dependent environmental changes, though to be less sensitive to the differences seen by TMA-DPH in all PA/PC vesicles, i.e., to effects nearer the headgroup region. PA served to rigidize the membrane in PC/PA sonicated vesicles, where steric constraints are significant and PA is located primarily in the inner leaflet, while PA served to fluidize the membrane in the ≈90 nm vesicles prepared by injection of PC/PA, where steric constraints are less significant and PA is presumed to be symmetrically distributed (cf. 141). Generally, trends noted in the presence of PA were more pronounced in the large diameter vesicle than in the small. Finally, the headgroup region of the outer leaflet of sonicated PC vesicles was noted to be more fluid than the corresponding region of injected PC vesicles.

These experiments were seen to be appropriate controls for measurements performed on vesicles with an asymmetric distribution of PA. As such, it would be necessary to repeat the experiments (i) with vesicles composed of ratios of PC/PA reflecting the possible distributions of PA in the vesicle product of the enzymatic hydrolysis, and (ii) with polarization values being recorded at

much smaller temperature intervals. More thorough analysis of the dependence of polarization on vesicle diameter, if desired, is possible by producing vesicles of different diameters by varying the concentration of phospholipid injected in the ethanol injection method (103, 117).

Valuable information on the impact of phospholipid asymmetry on the physical properties of membranes would be overlooked if studies were limited to probes which localize exclusively to the hydrophobic core of the membrane. DPH studies should continue to be complemented by experiments with TMA-DPH or another amphipathic probe. Interpretation of TMA-DPH polarization data requires further experimental work to establish that the distribution of the cationic TMA-DPH probe is random in leaflets containing PA. Multifrequency phase or energy transfer fluorescence measurements standard to this laboratory might be applicable to this question.

Alternatively, thought could given to other neutral amphipathic probes. Of some interest are the dipole-moment sensitive fluorophores related to prodan (Weber and co-workers, refs. 200, 119). Fluorescence emission spectra of the 6-lauroyl derivative of prodan (laurodan) in a variety of solvents establish it to be equally sensitive to solvent polarity. Simple organic synthetic methods are available to produce an amphipathic laurodan derivative with a hexose attached at the 2-position.

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